

**Appeal Brief**

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Dated: 09 OCTOBER 2009 Signature: /ALLEN E. WHITE/  
(Allen E. White)

Docket No.: HO-P03493US0  
(PATENT)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

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In re Patent Application of:  
Mrugesh Shah

Application No.: 10/776,711

Confirmation No.: 3444

Filed: February 12, 2004

Art Unit: 1637

For: Microorganisms producing petroleum  
from coal or hydrocarbons or from C, H or  
oxygen; producing C, H or oxygen from  
water or hydrocarbons

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Examiner: M. Staples

**APPEAL BRIEF**

MS Appeal Brief - Patents  
Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Dear Board of Patent Appeals:

As required under §41.37(c), this brief is filed within two months of the Notice of Appeal filed in this case on 28 August 2009, and is in furtherance of said Notice of Appeal.

The fees required under § 41.20(b)(2) are dealt with in the accompanying TRANSMITTAL OF APPEAL BRIEF.

This brief contains, under the appropriate headings and in the order indicated, the following items as required by 37 C.F.R. § 41.37(e):

- I. Statement of the Real Party In Interest
- II. Statement of Related Cases
- III. Jurisdictional Statement
- IV. Table of Contents
- V. Table of Authorities
- VI. Status of Amendments
- VII. Grounds of Rejection to be Reviewed
- VIII. Statement of Facts
- IX. Argument
- X. Appendix consisting of:
  - Claims
  - Claim Support And Drawing Analysis
  - Evidence
  - Related Cases

## **I. REAL PARTY IN INTEREST**

The real party in interest for this appeal is:

Mrugesh Shah, M.D.

## **II. RELATED CASES**

There are no other appeals, interferences, judicial proceedings or continuing applications or requests for continued examination which will directly affect or be directly affected by or have a bearing on the Board's decision in this appeal.

## **III. JURISDICTIONAL STATEMENT**

The Board has jurisdiction under 35 U.S.C. 134(a). The Examiner mailed a final rejection on 28 May 2009, setting a three-month shortened statutory period for response. The shortened statutory time for responding to the final rejection would have expired on 28 August 2009. Rule 134. A notice of appeal was filed on 28 August 2009. The time for filing an appeal brief is two months after the filing of a notice of appeal. Bd.R. 41.37(c). The time for filing an appeal brief would have expired on 28 October 2009. The appeal brief is being filed on 09 October 2009.

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## **VI. STATUS OF AMENDMENTS**

An amendment after final rejection filed 17 July 2009 has been entered upon by the Examiner. Claims 2, 4-8, 10 and 16 were canceled during prosecution. Claims 1, 3, 9, 11-15, 17 and 18 are pending, stand rejected and are all subject of the appeal.

## **VII. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL**

Rejection of claims 1, 3, 9, 11-15, 17 and 18 as failing to comply with the enablement requirement of the first paragraph of 35 U.S.C. § 112.

## **VIII. STATEMENT OF FACTS**

[01] Zaldivar, et. al. (“Zaldivar”; Exhibit A) is a reference describing the state of the art for commercial bioethanol production using a lignocellulose feedstock. *See, e.g.*, the Abstract; page 28, right column, last full paragraph.

[02] Zaldivar discloses several microorganisms capable of metabolizing lignocellulose derived sugars into bioethanol. *See*, pages 21-23 (*Broad substrate utilization range*).

[03] Zaldivar also describes successful metabolic engineering efforts to increase the efficiency of such utilization toward the goal of commercial production. *Id.*, summarized at Table 2.

[04] Zaldivar discusses an example of an unexpected failure of a genetic manipulation of a bioethanol microorganism where the result in industrial scale processes did not match that seen at the laboratory scale. *See* page 27, 2<sup>nd</sup> col., 1<sup>st</sup> full paragraph.

[05] Lin and Tanaka (“Lin”; Exhibit B) is a broad overview of bioethanol production from sugars, starches and cellulosic materials. *See* page 628, 1<sup>st</sup> col., 2<sup>nd</sup> paragraph.

[06] Jeffries and Jin (“Jeffries”; Exhibit C) is focused on metabolic engineering of yeast to utilize pentose sugars in bioethanol production to achieve commercial competitiveness of the process where lignocellulose is the feedstock. *See* Abstract.

[07] van Hamme et al. (“van Hamme”; Exhibit D) reviews the advanced state of knowledge on the genes and genetic circuits of alkane metabolizing microorganisms. *See* pages 506-514.

[08] The Application as filed directs one of skill in the art to exemplary microorganisms and culturing conditions found in the cited background reference WO0246446. Application as filed, page 1, lines 6-20.

[09] Kurashkov, et al. (“Kurashkov”; Exhibit E) discloses microorganisms capable of making biosynthetic petroleum and their culturing conditions. *See* Office action issued 22 Jan. 2007, page 15, 3<sup>rd</sup> paragraph.

[010] Martin S. Cohen and Peter D. Gabriele (“Cohen”; Exhibit F) show additional fungal microorganisms capable of converting coal into biosynthetic petroleum and their culturing conditions. *See, e.g.,* the Abstract.

[011] Applicant requests the Board take judicial notice of the existence of extensive technology for measuring and identifying the chemicals of petroleum. The Examiner refuses to acknowledge or concede that technology existed as of

the priority date for qualitative or quantitative measurements of petroleum compounds. Advisory action mailed 31 July 2009.

[012] The specification expressly identifies a subtraction hybridization/screening approach for isolating the genes. *See* page 3, line 19 to page 6, line 4 of the Application as filed.

[013] Expression screening and other standard molecular biology techniques for screening and isolating genes for proteins having specific biochemical activities were well established in the art as of the effective date of the Application. *See, e.g.,* King et al., page 1, 2<sup>nd</sup> paragraph (“King”; Exhibit G).

[014] The state of the art of synthetic biology, as it applies to microorganism based biofuels, was adequately developed as of the priority date of the instant application to enable one of ordinary skill in the art to transfect multiple genes to work together to produce complex biosynthetic pathways. *See* Kalscheuer, Abstract (Exhibit H); WO2007/0136762, Examples 2-8, Pages 51-57 (Exhibit I).

[015] The state of the art was enabling for both identification of microorganisms capable of producing biosynthetic petroleum and identification of the genes responsible for this activity. *See* Dennis; (Exhibit J).

[016] There was a post filing reduction to practice of the claimed subject matter. *See* Savage (Exhibit K); Ayres (Exhibit M); Plenty (Exhibit L).

[017] WO2007/0136762 corresponds the LS9 reduction to practice *supra*. See WO2007/0136762 (Exhibit I).

[018] WO2007/0136762 applies molecular biology and microbiology techniques which were within the skill in the art prior to the effective date of the Application. WO2007/0136762, Examples 2-8, Pages 51-57 (Exhibit I).

## IX.ARGUMENT

The Examiner's conclusion that the pending claims are not enabled should be reversed because it is based on the Examiner's erroneous contention that the claims must be enabled for an industrial scale and commercially viable means of production. No such requirement exists. MPEP § 2164; *CFMT, Inc. v. YieldUP International Corp.*, 349 F.3d 1333, 1338 (Fed. Cir. 2003). Applicant is not claiming a commercial scale biosynthetic petroleum manufacturing process. The claims are directed to a process for developing and optimizing recombinant microbiological strains with the capacity to biosynthesize petroleum, with the ultimate aspiration of using them in a commercially viable, large scale production capability.

Furthermore, the Examiner is supposed to outline what subject matter the Examiner deems enabled. MPEP § 2164.04 ("[T]he examiner should always look for enabled, allowable subject matter and communicate to applicant what that subject matter is at the earliest point possible in the prosecution of the application."). In this application, the Examiner has identified no such subject matter, industrial scale or otherwise. This is particularly vexing because Applicant has provided evidence showing reduction to practice of the claimed invention by others within a two year period after publication of this application. Nothing in this subsequent reduction to practice involved technology that was not readily available and well understood prior to Applicant's filing. The Examiner thus contends what has been done could not

have been done, commercial scale or otherwise.

A. Inappropriate Legal Standard {First presented in a response filed 20 July 2007, page 11.}

In order to make an Enablement rejection, the Examiner has the initial burden to establish a reasonable basis to question the Enablement provided for the claimed invention. MPEP § 2164.04; *In re Wright*, 999 F.2d 1557, 1562 (Fed. Cir. 1993). In the pending rejection, the Examiner alleges various *Wands* factors weigh against enablement and concludes from this analysis that the Specification does not enable the claims. The Examiner's analysis is flawed because it applies the wrong standard, i.e. the Examiner requires a successful reduction to practice on an industrial scale with commercial successful to satisfy the enablement requirement. MPEP § 2164; *CFMT, Inc. v. YieldUP International Corp.*, 349 F.3d 1333, 1338 (Fed. Cir. 2003).

The Examiner's imposition of a requirement for industrial scale production with commercial successful is represented by the following quotations from the prosecution history:



The state of the art at the time of the claimed invention is revealed by Zaldivar et al. (2001):

“However, keeping a realistic perspective is important. The improvement achieved in the fermentation step with the help of metabolic engineering is just one of the aspects of an integrated process. Hence, several pieces still remain to be properly assembled (and optimized) before an efficient industrial configuration is acquired. It is therefore anticipated that once in operation, the current model technologies will need several cycles of improvement/analysis, before optimization and competitiveness are achieved” (see last paragraph on p. 30).

First Office Action on the merits issued 22 Jan. 2007, page 9 (emphasis added).

Applicant first argues that Zaldivar et al. teach industrial configuration for ethanol production which is an old and well-developed technology. However, this old technology which is ethanol production from starting materials of sugar cane juice and cornstarch serves as comparison and backdrop for difficulties encountered in the new art of ethanol production from starting materials of the instant claimed invention, such as wheat straw (a type of grass or turf), corn stalks (a type of grass or turf), and forestry residues (a type of wood), see 2<sup>nd</sup> paragraph under Introduction on p. 17.

Final rejection issued 11 Dec. 2007, page 7. The “difficulties” the Examiner refers to are real. These are difficulties of economically viable, industrial scale ethanol production:

**In the production of bioethanol, several steps are interrelated: feedstock collection, transportation to the manufacturing center, preparation of the raw material, hydrolysis, fermentation, steam generation, product concentration (distillation), and waste disposal (Olsson and Hahn-Hägerdal 1996). These operations need to be fully optimized for an efficient ethanol plant. In Fig. 4, the current processes for crop-based ethanol production and a proposed model for lignocellulose-based ethanol production are compared.**

Zaldivar, page 28, right column, last full paragraph. The Examiner's extrapolation from the bioethanol literature is based on an erroneous enablement standard requiring industrial scale production and economic viability. The Examiner has nonetheless continued to explicitly apply this erroneous standard:

Applicant initially argues that the problems encountered in "commercially viable biofuel production" have no bearing on enablement of the instant claims. (Biofuel and fuel are interchanged throughout the following discussion.) First, the claims do not exclude "commercially viable biofuel production" which the instant specification attempts to achieve (see p. 6 lines 5-8). Thus such production is relevant to the instant claims.

Office Action 17 June 2008, pages 4-5, bridging paragraph. The Examiner has also applied the same erroneous standard to the Examiner's own cited art:

Examiner does not agree with Applicant's interpretation that Lin et al. enable fuel production from lignocellulose. Lin et al. conclude that "optimism is high" for such production and that Sweden "could" (and not "has") become self sufficient from such production.

Office Action 17 June 2008, pages 8. The Examiner's position is plainly contrary to the law which specifically precludes from the enablement standard

issues of industrial scale production and economic viability. MPEP § 2164; *CFMT, Inc. v. YieldUP International Corp.*, 349 F.3d 1333, 1338 (Fed. Cir. 2003).

### B. Detailed *Wands* Factor Analysis

If the Board agrees that the Examiner's standard of review for Enablement (requiring commercial success) is incorrect, the following detailed review of each *Wands* factor discussed by the Examiner is dispensable.

The Examiner has however argued that the evidence relied upon is relevant to various *Wands* factors. Applicant thus includes the following for the sake of completeness and to fully develop the record for further review.

#### 1. Nature of the invention and breadth of claims

Applicant concurs with the Examiner's characterization of this *Wands* factor.

2. Unpredictability of the Art and the State of The Art {First presented in a response filed 20 July 2007, page 10, last paragraph.}

The Examiner advances Zaldivar first as evidence of the unpredictability for obtaining microorganisms that have metabolic capabilities useful for biofuel production. Office Action issued 17 June 2008, page 6-7 (middle portion omitted). A careful reading of Zaldivar, however, shows the Examiner's interpretation of the reference is incorrect. The key term the Examiner overlooks in the quoted Zaldivar text is "efficiently." Statement of fact [01].

Contrary to the Examiner's analysis, Zaldivar discloses several microorganisms capable of metabolizing lignocellulose derived sugars into bioethanol. Statement of fact [02]. The paper also describes successful metabolic engineering efforts to increase the *efficiency* of such utilization toward the goal of commercial production. Statement of fact [03]. Because the Examiner misconstrues Zaldivar and this mistake is the foundation for the Examiner's conclusion that Zaldivar evidences the unpredictability of obtaining microorganisms that have metabolic capabilities useful for biofuel production, the Examiner's conclusion on this point should be rejected.

{Applicant first presented the next argument in a response filed 07 March, 2008, pages 14-15, section d.}

The Examiner relies upon Zaldivar for a second argument that the results of gene transfection in microorganisms are unpredictable. Final rejection issued 11 Dec 2007, page 9. Applicant again pointed out that the Examiner's contentions were erroneously predicated on a discussion of industrialization issues:

In the third case, the simultaneous utilization of maltose and glucose was investigated. The work done for this purpose serves to illustrate the challenges that metabolic engineering confronts when attempting the improvement of strain characteristics. In recombinant strains with an “industrial background,” the disruption in *MIG1* did not alleviate glucose repression, in contrast to laboratory strains (Klein et al. 1996, 1997, 1999). This undesirable and unexpected result emphasized that: (1) the cell is a complex network of regulatory mechanisms, just partially elucidated, which makes it difficult to predict the consequences of the genetic changes introduced; (2) efficient strategies for laboratory strains might not be adequate for industrial strains, since genetic background and genetic characteristics may be different; (3) for industrial purposes, it is important that the introduction of a desirable trait does not adversely affect characteristics such as high specific growth rate and low by-product formation (Olsson and Nielsen 2000).

Statement of fact [04]. The Examiner’s subsequent comments admit as much but again assert these industrial scale production issues are alleged to be applicable, thus, the Examiner applies an improper Enablement standard that requires a commercial reduction to practice. Office Action issued 17 June 2008, page 7. Because the Examiner’s basis for the unfavorable conclusion on this point is legally wrong, the Examiner’s conclusions on this point should be rejected.

{Applicant first presented the below argument in a response filed 07 March, 2008, pages 15-17, section e.}

The next major point the Examiner asserts relates to Van Hamme:

Hamme et al. speaks on the processes explicitly: “. . . the specifics of individual systems and the diversity of systems are yet to be fully described . . .” as provided by Applicant. As given in the last Office action, Hamme et al. maintain that hydrocarbon metabolism needs more basic work in enzymology and protein biochemistry in order to be understood. The claimed invention is to an operable system, that is to produce complex fuels, where the art teaches that such systems for n-alkanes, being but one component of a such a complex fuel, are not known fully described and not adequately understood.

Office Action issued 17 June 2008, page 8. It seems that the Examiner’s position here is that the alkane metabolism genes taught in Van Hamme are not enabled for their use because science has not fully characterized the enzymology of the gene products.

Two possible explanations exist for the Examiner’s position, neither of which has legal or factual merit. One is that the Examiner has made a legal mistake on a hard legal issue to fully grasp. One does not need to know how or why something works to patent it. One simply has to enable the practice of the claimed subject matter. MPEP § 2164.01. Alternatively, the Examiner presumes a detailed understanding of enzyme kinetics would be a necessary predicate to practicing the claimed processes. If so, the Examiner has not provided any explanation of why this is expected to be the case.

3. Quantity of Experimentation {First presented in a response filed 20 July. 2007, page 12, 1<sup>st</sup> paragraph.}

The Examiner’s initial analysis of this *Wands* factor amounted to an

outline of various “parameters which would have to be studied to apply this technology....” and an unsupported assertion that work on the listed parameters would require “years of inventive effort.” Office Action dated 22 Jan. 2007, pg 10. The Examiner then further alleged in support of the initial analysis that the Examiner’s cited art of Zaldivar, Lin, Jeffries and Van Hamme “each teach the difficulties of making just one synthetic component of coal or petroleum...” Final Office Action 11 Dec. 2007, page 12. As the Examiner clearly comprehended from the office action descriptions of these foregoing references, none discloses making a “component of coal or petroleum.” Zaldivar, Lin and Jeffries discuss specifically the challenges of commercial ethanol production using lignocellulose materials as the feedstock. Statement of facts [01], [05] and [06], respectively. The Examiner does not allege that ethanol is a component of petroleum. The Examiner’s conclusion of “years of inventive effort” derived from these references may be an argument by analogy. It is irrelevant regardless because these references again relate to industrial production in an economically viable manner. This is again the wrong standard and thus any argument by analogy the Examiner may be attempting is irrelevant to the issue of Enablement. The Examiner’s final comment on this *Wands* factor is that Applicant is wrong because the cited references do not have the word “commercial,” or an analogous term, in their titles. Office Action issued 17 June 2008, page 9-10. Applicant invites the Board to peruse beyond the titles to at least the abstracts of the Examiner’s references. *See, e.g.,* Zaldivar

(“competitive lignocellulose ethanol plant”); Jeffries (“commercialization seems feasible for some applications”).

{Applicant first presented the below argument in a response filed 07 March, 2008, pages 15-17, section e.}

The van Hamme reference is of a different nature. In particular, van Hamme is cited for its discussion of microbial alkane degradation metabolism. Office Action issued 17 June 2008, page 8. This is still not a teaching of *making* a component of coal or petroleum. Statement of fact [07]. In fairness, the Examiner may again mean that van Hamme shows by analogy that the work required to practice the claimed methods would be extensive. But here too the Examiner is simply off point. As discussed above under the Unpredictability heading, what the Examiner cites and relies upon is as irrelevant as the commercialization oriented materials of the other references.

#### 4. Working Examples

No working example is provided in the Specification. Working examples are not mandatory and nonenablement cannot be predicated solely on their absence. MPEP § 2164.02; *Gould v. Quigg*, 822 F.2d 1074, 1078 (Fed. Cir. 1987).

#### 5. Guidance in the Specification

The Examiner’s analysis of this *Wands* factor for the rejection of claims 17 and 18 consolidates the Examiner’s prior arguments concisely. Final Office action issued 28 May 2009, page 12-13. Applicant has addressed substantively



similar points first in a response filed 20 July 2007, pages 12, bridging paragraph to page 14, 1<sup>st</sup> full paragraph (for clarity, some contested issues addressed in this cited response are not relied upon by the Examiner in the currently appealed rejection).

Applicant presents the below argument for the first time in response to the herein appealed Final Office action issued 28 May 2009.

Most of the rejection of claims 17 and 18 are redundant with prior grounds of rejection addressed above. The Examiner further errs in the rejection of claims 17 and 18 by concluding that “the specification provides inadequate guidance to identify the gene of the starting organism that would need to be transfected.” The Examiner’s contentions are again premised on legal error. Enablement is judged by the disclosure plus the knowledge in the art. MPEP § 2164.01. *In re Wallach*, 378 F.3d 1330, 1333-34 (Fed. Cir. 2004). The *Specification* does not have to provide any guidance for things readily available and well known to those of ordinary skill in the art. For example, the Application refers to an international patent application and some of the specific microorganisms disclosed therein. Statement of fact [08]. This is not incorporated by reference and the technical details from it are not reproduced in the instant Application. Likewise, Applicant’s other reference on point and the art cited by the Examiner, discussed further below, are not even cited in the Application. *See* Kurashkov (Exhibit E); Cohen (Exhibit F). Nor does the Application reproduce the well know methods of expression cloning

(Exhibit G) or text books on petroleum chemistry showing various gas phase chromatography, mass spectroscopy techniques, etc., for identifying the presence and structures of the chemicals of petroleum. Statement of fact [11]. Things such as the Handbook of Petroleum Product Analysis, By: Speight, James G. © 2002, John Wiley & Sons (**409 pages**) need not be reproduced and are better off left out to avoid Specifications several thousands of pages long of things already known and readily accessible to those of skill in the art. See, e.g., *In re Myers*, 410 F.2d 420, 424 (C.C.P.A. 1969) (“A specification is directed to those skilled in the art and need not teach or point out in detail that which is well-known in the art.”); *Falkner v. Inglis*, 448 F.3d 1357, 1365 (Fed. Cir. 2006).

It is not Applicant’s burden to prove Enablement through evidence of the state of the art absent a reasonable basis to question enablement. MPEP 2164.04 and 2164.05; *In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971); *In re Brandstadter*, 484 F.2d 1395, 1406-07 (CCPA 1973). It is not a reasonable basis to allege that Applicant did not reproduce volumes of various background information in the body of the Specification. *In re Myers*, 410 F.2d 420, 424 (C.C.P.A. 1969). Nonetheless, Applicant made a good faith effort to provide the Examiner with relevant materials such as the Cohen reference and to remind the Examiner of the contents of his own Kurashkov reference. See Kurashkov (Exhibit E); Cohen (Exhibit F). The Examiner cannot base nonenablement by simply alleging that the information representing

the state of the art are not reproduced in the four corners of the Specification. Advisory Action, mailed 31 July 2009; MPEP § 2164.01. *In re Wallach*, 378 F.3d 1330, 1333-34 (Fed. Cir. 2004).

#### 6. Level of Skill in the Art

Applicant agrees that the level of ordinary skill in the art is very high. The skilled artisan will have a sophisticated understanding and complex skill set with which the artisan may reduce the claimed methods to practice without undue experimentation.

#### 7. Conclusion

The Examiner summarizes the Examiner's foregoing assessments and not surprisingly pronounces the claimed methods non-enabled. Because the Examiner did not properly analyze the foregoing *Wands* factors, the Examiner has not provided a reasonable basis to question Enablement by the Specification. Applicant requests the rejection be reversed.

C. Applicant Evidence of Enablement {First presented in a response filed 05 Sept. 2008, page 8 to page 14, top bridging paragraph.}

Applicant contends that the Board should reverse the rejection because the Examiner has applied a wrong standard for enablement and otherwise failed to apply the *Wands* factors appropriately. Because it is the Examiner's burden to establish unpatentability, the Examiner's failure to do so is dispositive. MPEP

2164.04; *In re Wright*, 999 F.2d 1557, 1562 (Fed. Cir. 1993). Applicant has gone beyond this to provided the Examiner with affirmative proof of enablement. This evidence is reproduced herein for the benefit of the Board and to create a complete record for further review.

### 1. Claim 1

Claim 1 is directed to a method of converting (i) solid fossil fuels, or (ii) oil tars obtained by distillation of coal, turf, grass, rubber, sapropel, sapropelites, slates, or wood, into biosynthetic petroleum, comprising the steps of:

a) isolating a starting microorganism capable of said conversion;

This step is what is commonly referred to as bioprospecting. The famous example of *Taq* Polymerase is a result of this type of activity. Examples of the successful practice of this step may also be found in Kurashkov, et al. cited by the Examiner and the specific species of microorganisms identified in claim 3 of the application. The art also demonstrates enablement of assays for whether a microorganism is capable of converting solid fossil fuels to biosynthetic petroleum. Statements of fact [09] and [010]. The Examiner contends in response to this evidence that this limitation is not enabled because “there is no known microorganism capable of ‘said conversion’ as recited.” Final Office Action issued 28 May 2009, page 4. The issue is whether the process step of ascertaining such microorganisms is enabled. Applicant respectfully submits the example microorganisms recited in claim 3 and identified by Kurashkov and

Cohen demonstrate the answer is yes. Statements of fact [09] and [010]. These also refute the Examiner's argument that no such microorganism is known. *Id.*; See also Claim 3 and the related disclosure in the Application as filed.

b) isolating from the starting microorganism the genes responsible for the conversion ability;

The next step in the claimed process involves cloning of the genes responsible for the identified microorganisms' ability to convert solid fossil fuels to biosynthetic petroleum. The specification expressly identifies a subtraction hybridization/screening approach for isolating the genes. Statement of fact [012]. Expression cloning and screening and other standard molecular biology techniques for screening and isolating these genes were well established in the art. Statement of fact [013]. Having isolated microorganisms with the desired biological activities, the process step of cloning the genes behind that activity represents the application of well established molecular biology techniques.

The Examiner's response to this is that a) one cannot perform the recited step solely using subtraction hybridization techniques and b) there is no guidance on the substep of moving from an enriched differential cDNA library to the identification of the responsible genes. Final Office Action issued 28 May 2009, page 4. The Examiner, however, overlooked Applicant's supplied expression cloning paper, which establishes that this type of molecular biology

work was routine, in the extreme, at the filing date of the application. This is of course especially true in the case of microorganism genetics. As such, this basic background knowledge need not, was not, and indeed should not have been reproduced in the application. See, e.g., *In re Myers*, 410 F.2d 420, 424 (C.C.P.A. 1969) (“A specification is directed to those skilled in the art and need not teach or point out in detail that which is well-known in the art.”); *Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co.*, 730 F.2d 1452, 1463 (Fed. Cir. 1984) (“The question is whether the disclosure is sufficient to enable those skilled in the art to practice the claimed invention, hence the specification need not disclose what is well known in the art.”); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384 (Fed. Cir. 1986) (“[A] **patent need not teach, and preferably omits, what is well known in the art.**”) (emphasis added); *Spectra-Physics, Inc. v. Coherent, Inc.*, 827 F.2d 1524, 1534 (Fed. Cir. 1987) (quoting *Hybritech*); *Falkner v. Inglis*, 448 F.3d 1357, 1365 (Fed. Cir. 2006) (quoting *Spectra-Physics*).

c) transfecting the genes into a host microorganism, and

The Examiner’s issues with this step of the claimed process appear to break down into two discrete subjects. At first, the Examiner questioned the ability in the state of the art to generate transgenic microorganism hosts generally. Office action issued 17 June 2008, page 12. This position seems to have given way to an argument that constructing artificial metabolic pathways

would require transecting multiple genes which would have to be expressed appropriately in the transgenic microbe. Office action issued 17 June 2008, page 12; Final Office Action issued 28 May 2009, page 5. While the Examiner is not particularly detailed on this point, the issue the Examiner raises appears to be the state of the art of synthetic biology as it relates to the claimed process step. The state of the art of synthetic biology as it applies to microorganism based biofuels was adequately developed as of the priority date of the instant application to enable one of ordinary skill in the art to transfect multiple genes to work together to produce complex biosynthetic pathways. Statement of fact [014]. These examples of synthetic biology applied to microorganism based biofuel production demonstrate the state of the art was both enabling for one of ordinary skill in the art and was fully enabled to execute step c) as claimed. As with the expression cloning reference above, the Examiner failed to properly address, much less distinguish, such evidence. Final Office Action issued 28 May 2009, page 5.

d) combining the host microorganism with the solid fossil fuels or oil tars under conditions suitable for the conversion of the solid fossil fuels or oil tars into biosynthetic petroleum.

The foregoing identified references also demonstrate the advanced state of the art with regard to optimizing microbiological culturing conditions. This general state of the art has also been successfully applied to define conditions

suitable for the conversion of the solid fossil fuels into biosynthetic petroleum. Statements of fact [09] and [010]. Each specific embodiment of transgenic host and transgene(s) will require some experimental work to define optimum culture conditions. This does not represent undue experimentation, but rather routine work that one skilled in the art would do to optimize conditions.

## 2. Claim 11

Claim 11 is directed to A method of converting carbon, hydrogen and oxygen into-biosynthetic coal or synthetic petroleum, comprising the steps of:

- (a) isolating a starting microorganism capable of said conversion;
- (b) isolating from the starting microorganism the genes responsible for the conversion ability;
- (c) transfecting the genes into a host microorganism; and
- (d) combining the host microorganism with the carbon, hydrogen and oxygen under conditions suitable for the conversion of the carbon, hydrogen and oxygen into biosynthetic coal or biosynthetic petroleum.

With regard to claim 11, the enabling state of the art for the claimed method is established by analogy to the evidence and explanation for claim 1. *See above.*

In addition, Applicant cites more evidence for claim 11, steps a) and b). The Dennis reference further demonstrates the enabling state of the art for both identification of microorganisms capable of producing biosynthetic petroleum



and identification of the genes responsible for this activity. Statement of fact [015].

For claim 11, steps c) and d), the success of LS9 in making biosynthetic petroleum is a direct example of applying synthetic biology to create transgenic microorganism hosts and then determining conditions suitable for producing biosynthetic petroleum. Statement of fact [016]. LS9 was formed in 2005, shortly after publication placed Applicant's invention in the public domain. Statement of fact [016]. One aspect of LS9's biofuels program was "to use synthetic biology to develop microorganisms that produce biofuels." Statement of fact [016]. As of the June 6, 2007 publication date of LS9-A, "LS9 microbes produce and excrete hydrocarbons that are useful as fuels. This demonstrates that the process the Examiner characterizes as nonenabled, was successfully done. At least by May of 2008, LS9 had optimized the biosynthetic petroleum composition to "about ten" alkanes species. Statement of fact [016]. "The company's oil will have the molecular diversity necessary to make a number of fuels and petroleum products but will be free of unwanted chemicals that can muck up engines." Statement of fact [016]. The top U.S. government expert in this area confirms the general enablement of the claimed processes. Statement of fact [016]. Applicant contends this is an example of the state of the art as of the filing date of the instant application and that the instant disclosure was thus within the capacity of one of ordinary skill in the art. Statements of fact [017] and [018]. Nothing in LS9's reduction to practice

involved the application of technologies created after the instant application's filing date. *Id.* What was lacking in the art was the invention itself, the conception of the invention. *Cf.* “ ‘We certainly have gone beyond what we think anybody else was even thinking of doing’ in terms of producing hydrocarbons from microbes, says George Church, a geneticist at Harvard Medical School and one of LS9's two founders.” Statement of fact [016].

#### D. Conclusion

Because the Examiner's Enablement rejection is premised on an incorrect standard for enablement and an erroneous analysis of the *Wands* factors, The Examiner's conclusion with regard to prior art is that the instant claims are patentable. Final Office action issued 28 May 2009, page 13. Applicant thus requests the Board reverse the Enablement rejection and remand with recommendation for allowance.

The appeal fees are filed herewith. Applicant believes no other fee is due with this response. However, if a fee is due, please charge our Deposit Account No. 06-2375, under Order No. HO-P03493US0 from which the undersigned is authorized to draw.

Dated: 09 October 2009

Respectfully submitted,

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## **X. APPENDIX**

### **CLAIMS**

A complete listing of the claims follows.

1. (rejected) A method of converting (i) solid fossil fuels , or  
(ii) oil tars obtained by distillation of coal, turf, grass, rubber, sapropel, sapropelites, slates, or wood, into biosynthetic petroleum, comprising the steps of:
  - a) isolating a starting microorganism capable of said conversion;
  - b) isolating from the starting microorganism the genes responsible for the conversion ability;
  - c) transfecting the genes into a host microorganism, and
  - d) combining the host microorganism with the solid fossil fuels or oil tars under conditions suitable for the conversion of the solid fossil fuels or oil tars into biosynthetic petroleum.
2. (Canceled)
3. (rejected) The method of claim 1 wherein the starting microorganism is Thiobacillus aquaesulis 4255 or 389, Thiosphaera pantotropha 356, Thiosphaera pantotropha 2944, Thoibacillus thoiparus 55, or mutants or variants thereof, or a microorganism which exists naturally in water.
4. – 8. (Canceled)
9. (rejected) A method of improving conversion of (i) solid fossil fuels, or  
(ii) oil tars obtained by distillation of coal, turf, grass, rubber, sapropel,

sapropelites, slates, or wood, into biosynthetic petroleum, comprising the steps of:

- (a) isolating a starting microorganism capable of said conversion;
- (b) isolating from the starting microorganism an oligonucleotide probe complementary to a gene responsible for the conversion ability;
- (c) placing the probe under hybridizing conditions in contact with amplicons from other microorganisms suspected to be capable of or being capable of said conversion;
- (d) isolating amplicons which hybridized;
- (e) transfecting the isolated amplicons into a host microorganism;
- f) combining the host microorganism with the solid fossil fuels or oil tars under conditions suitable for the conversion of the solid fossil fuels or oil tars into biosynthetic petroleum; and
- (g) determining whether productivity improved.

10. (Canceled)

11. (rejected) A method of converting carbon, hydrogen and oxygen into biosynthetic coal or biosynthetic petroleum, comprising the steps of:

- (a) isolating a starting microorganism capable of said conversion;
- (b) isolating from the starting microorganism the genes responsible for the conversion ability;
- (c) transfecting the genes into a host microorganism; and
- (d) combining the host microorganism with the carbon, hydrogen and

oxygen under conditions suitable for the conversion of the carbon,  
hydrogen and oxygen into biosynthetic coal or biosynthetic petroleum.

12. (rejected) The method of claim 11 wherein, after transfection, the transfected host microorganism as compared to the starting microorganism is capable of faster growth, reproduction, enhanced survivability in a production environment, or more production of biosynthetic coal or biosynthetic petroleum per unit of a nutrient.
13. (rejected) The method of claim 11 wherein the host microorganism can exist in salt water or fresh water, can metabolize glucose, rubber, grass, or other nutrient media, can survive acidic or basic environments, can oxidize sulfur, or can exist in aerobic or anaerobic conditions.
14. (rejected) The method of claim 11 wherein the genes responsible for conversion are isolated by subtractive hybridization.
15. (rejected) The method of claim 14 wherein the subtractive hybridization is performed by representational difference analysis.
16. (rejected) The method of claim 15 wherein before transfection, the genes are selectively altered, and following transfection with such selectively altered genes, the host microorganisms with characteristics best suited to commercial production of biosynthetic coal or biosynthetic petroleum are selected.
17. (rejected) A method of converting (i) solid fossil fuels, or  
(ii) oil tars obtained by distillation of coal, turf, grass, rubber, sapropel,

sapropelites, slates, or wood,

into biosynthetic petroleum, comprising the steps of:

- a) obtaining a gene encoding a protein capable of said conversion;
- b) transfecting the gene into a host microorganism, and
- c) combining the host microorganism with the solid fossil fuels or oil tars under conditions suitable for the conversion of the solid fossil fuels or oil tars into biosynthetic petroleum.

18. (rejected) A method of converting carbon, hydrogen and oxygen into biosynthetic coal or biosynthetic petroleum, comprising the steps of:

- a) obtaining a gene encoding a protein capable of said conversion;
- b) transfecting the gene into a host microorganism; and
- c) combining the host microorganism with the carbon, hydrogen and oxygen under conditions suitable for the conversion of the carbon, hydrogen and oxygen into biosynthetic coal or biosynthetic petroleum.

## CLAIM SUPPORT AND DRAWING ANALYSIS

The references to the Specification are provided as examples, and should not be considered an exhaustive list of support for the claim. Support can also be found in the claims as filed.

1. (rejected) A method of converting (i) solid fossil fuels , or (ii) oil tars obtained by distillation of coal, turf, grass, rubber, sapropel, sapropelites, slates, or wood, into biosynthetic petroleum {**Page 1, lines 6-11; page 2, lines 6-9; page 7, lines 13-15; original claim 1**}, comprising the steps of:

- a) isolating a starting microorganism capable of said conversion {**Page 1, lines 6-11; page 1, line 13 – page 2, line 3; page 4, lines 4-10; page 7, lines 13-15; original claim 1**};
- b) isolating from the starting microorganism the genes responsible for the conversion ability; {**Page 1, lines 6-11; page 2, lines 9-10; page 3, lines 10-17 and Fig. 1 (entire); page 3, line 19-page 6, line 4; original claim 1**}
- c) transfecting the genes into a host microorganism, {**Page 1, lines 11-13; page 2, lines 11-13; page 3, lines 6-8; page 6, lines 5-9; original claim 1**} and
- d) combining the host microorganism with the solid fossil fuels or oil tars under conditions suitable for the conversion of the solid fossil fuels or oil tars into biosynthetic petroleum {**Page 1, lines 6-11; page 1, lines**



**13-19; page 2, lines 13-14; original claim 4}.**

9. (rejected) A method of improving conversion of (i) solid fossil fuels, or  
(ii) oil tars obtained by distillation of coal, turf, grass, rubber, sapropel,  
sapropelites, slates, or wood, into biosynthetic petroleum, **{original claim 9}**  
comprising the steps of:

(a) isolating a starting microorganism capable of said conversion; **{Page 1, lines 6-11; page 1, line 13 – page 2, line 3; page 4, lines 4-10; page 7, lines 13-15; original claim 1}**

(b) isolating from the starting microorganism an oligonucleotide probe complementary to a gene responsible for the conversion ability **{Page 6, line 22-page 7, line 3}**

(c) placing the probe under hybridizing conditions in contact with amplicons from other microorganisms suspected to be capable of or being capable of said conversion; **{Page 7, lines 4-8}**

(d) isolating amplicons which hybridized; **{Page 7, lines 8-11}**

(e) transfecting the isolated amplicons into a host microorganism; **{Page 7, lines 8-11}**

f) combining the host microorganism with the solid fossil fuels or oil tars under conditions suitable for the conversion of the solid fossil fuels or oil tars into biosynthetic petroleum; **{Page 7, lines 8-11}** and

(g) determining whether productivity improved. **{Page 7, lines 8-11}**

11. (rejected) A method of converting carbon, hydrogen and oxygen into biosynthetic coal or biosynthetic petroleum **{original claim 11}**, comprising the steps of:

(a) isolating a starting microorganism capable of said conversion

**{original claim 11}**;

(b) isolating from the starting microorganism the genes responsible for the conversion ability **{Page 1, lines 6-11; page 2, lines 9-10; page 3, lines 10-17 and Fig. 1 (entire); page 3, line 19-page 6, line 4; original claim 11}**;

(c) transfecting the genes into a host microorganism; **{Page 1, lines 11-13; page 2, lines 11-13; page 3, lines 6-8; page 6, lines 5-9; original claim 11}** and

(d) combining the host microorganism with the carbon, hydrogen and oxygen under conditions suitable for the conversion of the carbon, hydrogen and oxygen into biosynthetic coal or biosynthetic petroleum **{Page 3, lines 1-4; original claim 11}**.

17. (rejected) A method of converting (i) solid fossil fuels, or (ii) oil tars obtained by distillation of coal, turf, grass, rubber, sapropel, sapropelites, slates, or wood, into biosynthetic petroleum **{Page 1, lines 6-11; page 2, lines 6-9;**

**page 7, lines 13-15; original claim 1}**, comprising the steps of:

- a) obtaining a gene encoding a protein capable of said conversion **{Page 1, lines 6-11; page 1, line 13 – page 2, line 3; page 2, lines 9-10; page 3, lines 10-17 and Fig. 1 (entire); page 3, line 19-page 6, line 4; page 7, lines 13-15; original claim 1}**;
- b) transfecting the gene into a host microorganism, **{Page 1, lines 11-13; page 2, lines 11-13; page 3, lines 6-8; page 6, lines 5-9; original claim 1}** and
- c) combining the host microorganism with the solid fossil fuels or oil tars under conditions suitable for the conversion of the solid fossil fuels or oil tars into biosynthetic petroleum **{Page 1, lines 6-11; page 1, lines 13-19; page 2, lines 13-14; original claim 4}**.

18. (rejected) A method of converting carbon, hydrogen and oxygen into biosynthetic coal or biosynthetic petroleum **{original claim 11}**, comprising the steps of:

- a) obtaining a gene encoding a protein capable of said conversion **{Page 1, lines 6-11; page 2, lines 9-10; page 3, lines 10-17 and Fig. 1 (entire); page 3, line 19-page 6, line 4; original claim 11}**;
- b) transfecting the gene into a host microorganism; **{Page 1, lines 11-13; page 2, lines 11-13; page 3, lines 6-8; page 6, lines 5-9; original claim 1}** and

c) combining the host microorganism with the carbon, hydrogen and oxygen under conditions suitable for the conversion of the carbon, hydrogen and oxygen into biosynthetic coal or biosynthetic petroleum {Page 3, lines 1-4; original claim 11}.

## **EVIDENCE**

Pursuant to § 41.37(t) evidence upon which the appellant relied before the examiner and evidence relied upon by the appellant and admitted into the file pursuant to § 41.33(d) is attached hereto.

### **Table of Contents**

**Final Office action** mailed 28 May 2009.

**Advisory action** mailed 31 July 2009.

**Examiner References – Final Office action** (only those not relied upon by Applicant as Exhibits A-M are listed):

1. Duga et al., The intron-containing L3 ribosomal protein gene (RPL3): sequence analysis and identification of U43 and of two novel intronic small nucleolar RNAs, *Biochimica et Biophysica Acta* 1490 (2000) 225-236.
2. Pesole, Review, What is a gene? An updated operational definition, *Gene* 417 (2008) 1-4.
3. The American Heritage® Dictionary of the English Language: Fourth Edition. 2000, "petroleum", retrieved 2009.
4. coal. ( 2009). In *Encyclopcedia Britannica*. Retrieved May 24, 2009, from *Encyclopcedia Britannica Online*:  
<http://www.search.eb.com/eb/article-9110442>

5. Izizaki et al., Microbial production of poly-D-3-hydroxybutyrate from CO<sub>2</sub>, Appl Microbiol Biotechnol (2001) 57:6-12.
6. Cloning of the Alcaligenes latus Polyhydroxyalkanoate Biosynthesis Genes and Use of These Genes for Enhanced Production of Poly(3-hydroxybutyrate) in Escherichia coli, APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1998, p. 4897-4903.

Exhibits A-C and E were cited by the Examiner on a USPTO Form PTO-892 dated January 22, 2007. Exhibit D was cited by the Examiner on a USPTO Form PTO-892 dated December 11, 2007. Exhibits F-M were cited by Appellant on an IDS dated September 05, 2008.

**EXHIBIT A** Zaldivar, et al., Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration; Applied Microbiology and Biotechnology, Volume 56, Numbers 1-2 / July, 2001, pages 17-34.

**EXHIBIT B** Yan Lin and Shuzo Tanaka, Ethanol fermentation from biomass resources: current state and prospects, Applied Microbiology and Biotechnology, Volume 69, Number 6 / February, 2006, Pages 627-642.

**EXHIBIT C** Jeffries, T. W., Jin, Y. S., Metabolic engineering for improved fermentation of pentoses by yeasts, Applied microbiology and biotechnology. Vol. 63 (2004): Pages 495-509.

- EXHIBIT D** van Hamme JD, Singh A, Ward OP (2003) Recent advances in petroleum microbiology. *Microbiol Mol Biol Rev* 67:503–549.
- EXHIBIT E** Kurashkov, et al., RU-2180919 C1 (English translation by USPTO).
- EXHIBIT F** Martin S. Cohen and Peter D. Gabriele, Degradation of Coal by the Fungi *Polyporus versicolor* and *Poria monticola*, *Appl Environ Microbiol.* 1982 July; 44(1): 23-27.
- EXHIBIT G** King RW, Lustig KD, Stukenberg PT, McGarry TJ, Kirschner MW. Expression cloning in the test tube. *Science.* 1997 Aug 15;277(5328):973-4.
- EXHIBIT H** Kalscheuer R, Stölting T, Steinbüchel A. Microdiesel: *Escherichia coli* engineered for fuel production. *Microbiology.* 2006 Sep;152(Pt 9):2529-36.
- EXHIBIT I** WO2007/0136762
- EXHIBIT J** Dennis M, Kolattukudy PE. A cobalt-porphyrin enzyme converts a fatty aldehyde to a hydrocarbon and CO. *Proc Natl Acad Sci U S A.* 1992 Jun 15;89(12):5306-10.
- EXHIBIT K** Neil Savage, Building Better Biofuels, *Technology Review* (MIT), June 06, 2007.
- EXHIBIT L** Chris Ayres, Scientists find bugs that eat waste and excrete petrol, *The TimesOnline*, June 14, 2008.

**EXHIBIT M** Next-generation biofuels: nearly identical to gasoline and diesel, *Plenty* May 21, 2008.



## **RELATED PROCEEDINGS**

No related proceedings are referenced, hence copies of decisions in related proceedings are not provided.



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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
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10/776,711

02/12/2004

Mrugesh Shah

HO-P03493US0

3444

26271 7590 05/28/2009  
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EXAMINER

STAPLES, MARK

ART UNIT

PAPER NUMBER

1637

MAIL DATE

DELIVERY MODE

05/28/2009

PAPER

**Please find below and/or attached an Office communication concerning this application or proceeding.**

The time period for reply, if any, is set in the attached communication.

<b>Office Action Summary</b>	<b>Application No.</b> 10/776,711	<b>Applicant(s)</b> SHAH, MRUGESH	
	<b>Examiner</b> MARK STAPLES	<b>Art Unit</b> 1637	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

### Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

### Status

- 1) ☒ Responsive to communication(s) filed on 02 March 2009.
- 2a) ☒ This action is **FINAL**.                      2b) ☐ This action is non-final.
- 3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

### Disposition of Claims

- 4) ☒ Claim(s) 1,3,9 and 11-18 is/are pending in the application.
- 4a) Of the above claim(s) \_\_\_\_\_ is/are withdrawn from consideration.
- 5) ☐ Claim(s) \_\_\_\_\_ is/are allowed.
- 6) ☒ Claim(s) 1, 3, 9, and 11-18 is/are rejected.
- 7) ☐ Claim(s) \_\_\_\_\_ is/are objected to.
- 8) ☐ Claim(s) \_\_\_\_\_ are subject to restriction and/or election requirement.

### Application Papers

- 9) ☐ The specification is objected to by the Examiner.
- 10) ☐ The drawing(s) filed on \_\_\_\_\_ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.  
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).  
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).
- 11) ☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

### Priority under 35 U.S.C. § 119

- 12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
- a) ☐ All    b) ☐ Some \*    c) ☐ None of:
1. ☐ Certified copies of the priority documents have been received.
  2. ☐ Certified copies of the priority documents have been received in Application No. \_\_\_\_\_.
  3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

\* See the attached detailed Office action for a list of the certified copies not received.

### Attachment(s)

- |                                                                                        |                                                                   |
|----------------------------------------------------------------------------------------|-------------------------------------------------------------------|
| 1) <input checked="" type="checkbox"/> Notice of References Cited (PTO-892)            | 4) <input type="checkbox"/> Interview Summary (PTO-413)           |
| 2) <input type="checkbox"/> Notice of Draftsperson's Patent Drawing Review (PTO-948)   | Paper No(s)/Mail Date. _____                                      |
| 3) <input checked="" type="checkbox"/> Information Disclosure Statement(s) (PTO/SB/08) | 5) <input type="checkbox"/> Notice of Informal Patent Application |
| Paper No(s)/Mail Date <u>9/5/08</u> .                                                  | 6) <input type="checkbox"/> Other: _____                          |

### **DETAILED ACTION**

1. Applicant's amendment of claims 1, 9, 11, 12, and 16 and the submission of new claims 16 and 17 in the paper filed on 03/02/2009 is acknowledged.

Claims 1, 3, 9, and 11-18 are pending and at issue.

The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

### **Rejections that are Withdrawn**

#### ***Claim Rejections Withdrawn In Part - 35 USC § 112 First Paragraph***

2. The rejections of claims 1, 3, 9, and 11-16 in the part for reciting new matter of "synthetic petroleum" under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement is withdrawn. Applicant has amended the claims to recite "biosynthetic petroleum" in place of "synthetic petroleum". And while there is no literal support for the term "biosynthetic petroleum" in the originally filed application including the originally filed specification; the originally claimed methods recited combining a microorganism with recited material(s) to produce a product which would thus be biosynthetic, if accomplished. Thus the current amendments reciting "biosynthetic petroleum" are accepted.

***Claim Rejections Withdrawn - 35 USC § 112 Second Paragraph***

3. The rejections of claims 23 and 26 under 35 USC § 112 Second Paragraph are withdrawn in light of Applicant's removal of the word "plurality" from these claims and amendment to recite the number of primers supported by the specification.

***Claim Rejections Withdrawn - 35 USC § 112 Second Paragraph***

***Omitting Essential Elements***

4. The rejections of claims 1, 3, 9, and 11-15 under 35 U.S.C. 112, second paragraph, as being incomplete for omitting essential elements is withdrawn. Applicant has amended the claims to recite a step of conversion. However, this step is not found to be enabled as given in the following section.

***Rejections that are Maintained***

***Lack of Enablement***

5. The rejection of claims 1, 3, 9, and 11-16 under 35 U.S.C. 112, first paragraph, as failing to comply with the enablement requirement is maintained. Applicant's arguments filed 03/02/2009 have been considered but they are not persuasive.

***Cited References are Not Considered***

Applicant's arguments with respect to cited references are not considered here as the cited references have not been submitted in a proper Information Disclosure Statement (see ***Improper Information Disclosure*** below).

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*Claim 1*

Applicant argues that step a) of claim 1 is known in the art as “bioprospecting” and that Examiner appears to concur as this step does not appear to be specifically but generally challenged. As a matter of record, Examiner does not concur that step a) of claim 1 was known in the art either specifically or generally. The claim recites “said conversion” which is a conversion that is unknown in the art and where there is no known microorganisms capable of “said conversion” as recited. “Bioprospecting” for a heretofor unknown conversion and/or microorganism is not a matter of knowledge but a lack of guidance.

Applicant argues two points regarding step b) of claim 1. The gist of the first point is that Applicant need not provide the detailed processes of isolating the genes needed by subtractive hybridization as both gene isolation and subtractive hybridization were known in the art. However, Applicant is not applying either of these in a manner to enable the claimed invention. Subtractive hybridization merely points to genetic differences between organisms, as given in the last Office action. Applicant has not provided any guidance as to how to move from this to isolating the genes responsible for “said conversion”. This is also the guidance that is not known in the art, much less well known, and this is essential guidance that instant application does not provide. The second point argued is that the cited teaching of Hamme et al., regarding how alkane biosynthetic systems evolved, is misinterpreted. Hamme et al. is not referring to the process of evolution of organisms but to how the different approaches to alkane metabolism will need to evolve/change so that alkane metabolism might eventually be

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applied in chemical synthesis. In other words, Hamme et al. speak directly to the difficulty in just producing alkanes by microorganisms. Applicant claims to have a biosynthetic method for producing not just alkanes which are but a component of petroleum but for biosynthetic petroleum which is complex mixture of varied and multiple components.

Applicant argues that step c of claim 1 for transfecting genes is enabled. Applicant argues that transfection of a gene is known and thus the instant claims are enabled for transfecting. However, this is not the transfecting of the instant claims. Neither the instant applicant nor the art including the present state of the art enables the claims for transfecting genes (plural) the essential expressing of multiple genes acting in proper concert across multiple biosynthetic pathways to biosynthetically convert complex starting materials into a complex mixture which is biosynthetic petroleum.

Applicant argues that in step d the conditions of the biosynthetic conversion are known as conditions for synthetic conversion, i.e. without the use of microorganisms, are known. Applicant has not provided evidence the conditions for synthetic conversion are applicable to the conditions for biosynthetic conversion. In fact the art teaches otherwise as just the temperatures of biosynthetic conversion cannot be so high as to kill the microorganisms.

#### *Claim 11*

Applicant argues that recently the art teaches that natural microorganisms have been found which convert waste into petrol/petroleum. First, Applicant does not reveal

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what this "waste" is. Second, the claimed invention is not to microorganisms found in nature but to a host microorganism transfected by the genes responsible for the natural conversion.

Applicant argues the claims need not be enabled for commercial production since, with the exception of claim 16, there is no direct recitation of "commercial" in the claims. Applicant further argues that references cited by Examiner which mention or discuss commercial production among other things are not relevant to the instant claims and should be disregarded. Applicant then cites post filing date art which discusses commercial production and argues that these citations of Applicant are relevant and demonstrate that the instant claims are enabled. Thus Applicant's position appears to be conflicted. The crux is that petroleum is and for some time has been a commercial product. Thus it is to be expected that the art which discusses petroleum or biosynthetic petroleum production will mention commercial production at least in passing. This does not by itself make this art irrelevant even to those instant claims which do not recite "commercial". While a goal may be commercial production, the art cited by Examiner expressly discusses other subject matter relevant to the instant claims as given in previous Office actions.

Applicant specifically argues that since claim 16 recites "best suited to commercial production" that the claim is not related to commercial production. However the claim specifically directs the reader to commercial production and thus commercial production is relevant as recited positively in the claim as a claim limitation.



**New Objections and Rejections Necessitated by Amendment**

***Improper Information Disclosure***

6. The information disclosure statement filed on 09/05/2008 dispersed throughout Applicant Arguments/Remarks Made in Amendment fails to comply with the provisions of 37 CFR 1.97, 1.98 and MPEP § 609 because the content requirements as discussed in MPEP § 609.04(a) are not complied with and because copies of non-patent literature have not been provided. It has been placed in the application file, but the information referred to therein has not been considered as to the merits. Applicant is advised that the date of any re-submission of any item of information contained in this information disclosure statement or the submission of any missing element(s) will be the date of submission for purposes of determining compliance with the requirements based on the time of filing the statement, including all certification requirements for statements under 37 CFR 1.97(e). See MPEP § 609.05(a).

***New Claim Objections***

7. Claim 18 is objected to because of the following informalities: for recitation of "synthetic petroleum" in line 2 when it appears that "biosynthetic petroleum" is intended. Appropriate correction is required.

***New Claim Rejections - 35 USC § 112, First Paragraph***

8. The following is a quotation of the first paragraph of 35 U.S.C. 112:

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the

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art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

9. Claims 17 and 18 are rejected for reciting new matter of "obtaining a gene coding a protein capable of said conversion" under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The original specification and original claims describe genes (plural) capable of said conversion, and not a singular gene capable of said conversion. It is noted that the specification does refer to a probe to one of these genes and also refers to the gene of these genes which is responsible for the conversion, but the specification does not disclose the conversion as claimed as through one gene. Furthermore, neither the original specification nor the claims describe a gene encoding a protein much less a gene encoding a protein capable of the conversion. And it is noted that genes need not encode proteins, Duga et al. (2000) teach genes which do not encode proteins (see 2<sup>nd</sup> sentence of 5<sup>th</sup> paragraph on p. 233). And Pesole (2008) teaches that: "A large fraction of genes do not encode for proteins" (see particular point 1 on p. 2).

### ***Claim Interpretation***

10. As neither the claims nor the specification define the term "biosynthetic petroleum" this is reasonably interpreted to mean petroleum which is produced through biosynthesis, that is, through a process directly involving living organisms which can be microorganisms. American Heritage® Dictionary (retrieved 2009) and Encyclopaedia Britannica (retrieved 2009, "petroleum") are relied upon for definitions of petroleum. Synthetic petroleum is then reasonably interpreted to be a complex mixture containing

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multiple components found in petroleum and/or multiple components chemically similar to those found in petroleum.

11. As neither the claims nor the specification define the term "biosynthetic coal" this is reasonably interpreted to mean coal which is produced through biosynthesis, that is, through a process directly involving living organisms which can be microorganisms. Encyclopaedia Britannica (retrieved 2009, "coal") is relied upon for definitions of petroleum. Synthetic coal is then reasonably interpreted to be a complex mixture containing multiple components found in coal and/or multiple components chemically similar to those found in coal.

***New Claim Rejections - 35 USC § 112, First Paragraph***

***Lack of Enablement***

12. Claims 17 and 18 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the enablement requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

Factors to be considered in determining whether a disclosure meets the enablement requirement of 35 USC 112, first paragraph, have been described by the court in *In re Wands*, 8 USPQ2d 1400 (CA FC 1988). *Wands* states at page 1404,

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“Factors to be considered in determining whether a disclosure would require undue experimentation have been summarized by the board in *Ex parte Forman*. They include (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims.”

#### The nature of the invention and breadth of claims

Claims 17 and 18 are broadly drawn to methods of producing products being or resembling the complex and the multiple components of petroleum or coal.

#### The unpredictability of the art and the state of the prior art

There is a great deal of unpredictability in field of biofuel production. Ethanol production has received a lot of attention owing to the availability of renewable feedstock from biomass. This technology is directed to production of just one compound, ethanol. Yet there is considerable difficulty in making ethanol production a viable process. The state of the art at the time of the claimed invention is revealed by Zaldivar et al. (2001, previously cited):

“However, keeping a realistic perspective is important.

The improvement achieved in the fermentation step with the help of metabolic engineering is just one of the aspects of an integrated process. Hence, several pieces still remain to be properly assembled (and optimized) before an efficient industrial configuration is acquired. It is therefore anticipated that once in operation, the current model technologies will need several cycles of improvement/analysis, before optimization and competitiveness

are achieved” (see last paragraph on p. 30).

The post filing date art further confirms the unpredictability of this area. Jeffries et al. (2004, previously cited) convey: “However, the bioconversion of pentoses to ethanol still presents a considerable economic and technical challenge . . . .” (see 3<sup>rd</sup> sentence on p. 496). Lin et al. (2006, previously cited) disclose: “In this field, although bioethanol production has been greatly improved by new technologies, there are still challenges that need further investigations. A further understanding of the ethanol fermentation needs to be reached” (see 2<sup>nd</sup> column, 1<sup>st</sup> paragraph, last 2 sentences on p. 627). And: “Recently, research has concentrated on the development of improved processes; however, there are still challenges that need further investigations” (see 2<sup>nd</sup> sentence under the section *Most promising prospects* on p. 635).

#### Quantity of Experimentation

The quantity of experimentation in this area is extremely large since there is significant number of parameters which would have to be studied to apply this technology, including variability of the fossil fuels such as coal from source to source, ability of the host microorganism to adapt and convert these varies sources to products found in petroleum or coal, ability of the host microorganism to produce the multitude of compounds claimed, and the conditions necessary for the microorganism to produce those compounds. The time table necessary to achieve efficacious investigation of these parameters would require a very large quantity of experimentation. This would require years of inventive effort, with each of the many intervening steps, upon effective reduction to practice, not providing any guarantee of success in the succeeding steps.

Working Examples

The specification has no working examples of the claimed invention.

Guidance in the Specification.

The specification provides no evidence that a transfected host microorganisms would be able to produce the products found in petroleum or coal. The specification does not provide support that a host microorganism would be able to produce such products and that a starting microorganism would have a gene capable of such conversion. As found in Table 1 of Ishizaki et al. (2001, previously cited), the recombinant *E. coli* producing P(3HB) is not superior to two starting microorganisms *P. extroquens* and *R. eutropha*. Furthermore, Choi et al. (1998, previously cited) show that several recombinant *E. coli* did not adequately generate product, as shown in Figure 3. Thus just making a transfected host microorganism does not guarantee it will adequately produce the products as claimed. The guidance provided by the specification amounts to an invitation for the skilled artisan to try and follow the disclosed instructions to use the claimed invention. The specification merely discloses that one follow the complex procedure of gene identification and transfection followed by even the more complex procedure of selecting and optimizing growth conditions of the host microorganism to produce a vast arrays of distinct products by one cultivation of the host microorganism. Furthermore, the specification provides inadequate guidance to identify the gene of the starting organism that would need to be transfected. The products claimed to be produced are chemically more complex than ethanol and the expectation of the state of the art is that a complex metabolic pathway with several enzymes (and hence several genes of the starting microorganism) would be needed to yield the complex mixture of complex products. Thorough review of the prior art fails to

show any enabled teachings of a transfected microorganism producing the multitude of products found in petroleum and coal.

#### Level of Skill in the Art

The level of skill in the art is deemed to be high.

#### Conclusion

In the instant case, as discussed above, the factor of unpredictability weighs heavily in favor of undue experimentation in this highly unpredictable art where the variables of transfection of genes into microorganisms to produce biosynthetic petroleum or biowynthetic coal depend upon numerous parameters such as what panel of genes may be needed, how the feedstock to be converted into fuel is to be pretreated, how the transfected microorganism is to be introduced into the feedstock and how its growth is to be maintained, and what the acceptable limits are of temperature, pressure, aeration or lack of aeration, and degree of mixing. Further, the prior art and the specification provides insufficient guidance to overcome the art recognized problems in the use of producing the complete range of products found in petroleum or coal by a gene from one specie of a starting microorganism. Thus given the broad claims in an art whose nature is identified as unpredictable, the large quantity of research required to define the unpredictable variables, the lack of guidance provided in the specification, the absence of a working example, and the negative teachings in the prior art balanced only against the high skill level in the art, it is the position of the examiner that it would require undue experimentation for one of skill in the art to perform the method of the claim as broadly written.

**Conclusion**

13. No claim is allowed.

14. Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

15. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Mark Staples whose telephone number is (571) 272-9053. The examiner can normally be reached on Monday through Thursday, 9:00 a.m. to 6:00 p.m.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Benzion can be reached on (571) 272-0782. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.



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Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

Mark Staples

/M. S./

Examiner, Art Unit 1637

May 25, 2009

/Kenneth R Horlick/

Primary Examiner, Art Unit 1637



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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/776,711	02/12/2004	Mrugesh Shah	HO-P03493US0	3444
26271	7590	07/31/2009		
FULBRIGHT & JAWORSKI, LLP 1301 MCKINNEY SUITE 5100 HOUSTON, TX 77010-3095			EXAMINER STAPLES, MARK	
			ART UNIT 1637	PAPER NUMBER
			NOTIFICATION DATE 07/31/2009	DELIVERY MODE ELECTRONIC

**Please find below and/or attached an Office communication concerning this application or proceeding.**

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

twrye@fulbright.com  
aalvarez@fulbright.com  
nstacey@fulbright.com

<b>Advisory Action</b> <b>Before the Filing of an Appeal Brief</b>	<b>Application No.</b> 10/776,711	<b>Applicant(s)</b> SHAH, MRUGESH	
	<b>Examiner</b> MARK STAPLES	<b>Art Unit</b> 1637	

**--The MAILING DATE of this communication appears on the cover sheet with the correspondence address --**

THE REPLY FILED 17 July 2009 FAILS TO PLACE THIS APPLICATION IN CONDITION FOR ALLOWANCE.

1. ☒ The reply was filed after a final rejection, but prior to or on the same day as filing a Notice of Appeal. To avoid abandonment of this application, applicant must timely file one of the following replies: (1) an amendment, affidavit, or other evidence, which places the application in condition for allowance; (2) a Notice of Appeal (with appeal fee) in compliance with 37 CFR 41.31; or (3) a Request for Continued Examination (RCE) in compliance with 37 CFR 1.114. The reply must be filed within one of the following time periods:

- a) ☐ The period for reply expires \_\_\_\_\_ months from the mailing date of the final rejection.  
 b) ☒ The period for reply expires on: (1) the mailing date of this Advisory Action, or (2) the date set forth in the final rejection, whichever is later. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of the final rejection.

Examiner Note: If box 1 is checked, check either box (a) or (b). ONLY CHECK BOX (b) WHEN THE FIRST REPLY WAS FILED WITHIN TWO MONTHS OF THE FINAL REJECTION. See MPEP 706.07(f).

Extensions of time may be obtained under 37 CFR 1.136(a). The date on which the petition under 37 CFR 1.136(a) and the appropriate extension fee have been filed is the date for purposes of determining the period of extension and the corresponding amount of the fee. The appropriate extension fee under 37 CFR 1.17(a) is calculated from: (1) the expiration date of the shortened statutory period for reply originally set in the final Office action; or (2) as set forth in (b) above, if checked. Any reply received by the Office later than three months after the mailing date of the final rejection, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

#### NOTICE OF APPEAL

2. ☐ The Notice of Appeal was filed on \_\_\_\_\_. A brief in compliance with 37 CFR 41.37 must be filed within two months of the date of filing the Notice of Appeal (37 CFR 41.37(a)), or any extension thereof (37 CFR 41.37(e)), to avoid dismissal of the appeal. Since a Notice of Appeal has been filed, any reply must be filed within the time period set forth in 37 CFR 41.37(a).

#### AMENDMENTS

3. ☐ The proposed amendment(s) filed after a final rejection, but prior to the date of filing a brief, will not be entered because  
 (a) ☐ They raise new issues that would require further consideration and/or search (see NOTE below);  
 (b) ☐ They raise the issue of new matter (see NOTE below);  
 (c) ☐ They are not deemed to place the application in better form for appeal by materially reducing or simplifying the issues for appeal; and/or  
 (d) ☐ They present additional claims without canceling a corresponding number of finally rejected claims.

NOTE: \_\_\_\_\_. (See 37 CFR 1.116 and 41.33(a)).

4. ☐ The amendments are not in compliance with 37 CFR 1.121. See attached Notice of Non-Compliant Amendment (PTOL-324).  
 5. ☐ Applicant's reply has overcome the following rejection(s): \_\_\_\_\_.  
 6. ☐ Newly proposed or amended claim(s) \_\_\_\_\_ would be allowable if submitted in a separate, timely filed amendment canceling the non-allowable claim(s).  
 7. ☒ For purposes of appeal, the proposed amendment(s): a) ☐ will not be entered, or b) ☒ will be entered and an explanation of how the new or amended claims would be rejected is provided below or appended.  
 The status of the claim(s) is (or will be) as follows:  
 Claim(s) allowed: \_\_\_\_\_.  
 Claim(s) objected to: \_\_\_\_\_.  
 Claim(s) rejected: 1,3,9,11-15,17 and 18.  
 Claim(s) withdrawn from consideration: \_\_\_\_\_.

#### AFFIDAVIT OR OTHER EVIDENCE

8. ☐ The affidavit or other evidence filed after a final action, but before or on the date of filing a Notice of Appeal will not be entered because applicant failed to provide a showing of good and sufficient reasons why the affidavit or other evidence is necessary and was not earlier presented. See 37 CFR 1.116(e).  
 9. ☐ The affidavit or other evidence filed after the date of filing a Notice of Appeal, but prior to the date of filing a brief, will not be entered because the affidavit or other evidence failed to overcome all rejections under appeal and/or appellant fails to provide a showing of good and sufficient reasons why it is necessary and was not earlier presented. See 37 CFR 41.33(d)(1).  
 10. ☐ The affidavit or other evidence is entered. An explanation of the status of the claims after entry is below or attached.

#### REQUEST FOR RECONSIDERATION/OTHER

11. ☒ The request for reconsideration has been considered but does NOT place the application in condition for allowance because:  
See Continuation Sheet.  
 12. ☐ Note the attached Information *Disclosure Statement*(s). (PTO/SB/08) Paper No(s). \_\_\_\_\_.  
 13. ☐ Other: \_\_\_\_\_.

/Kenneth R Horlick/  
 Primary Examiner, Art Unit 1637

Continuation of 11. does NOT place the application in condition for allowance because:

Claim 16 has been canceled in the amendment filed 07/17/2009 which is entered. As claim 16 is canceled, the rejections and objections to claim 16 are moot and therefore are withdrawn. It is noted that Applicant presents arguments regarding canceled claim 16 but as claim 16 is canceled, these arguments are also moot.

The pending claims remain rejected as follows

The rejection of claims 1, 3, 9, 11-15, 17, and 18 under 35 U.S.C. 112, first paragraph, as failing to comply with the enablement requirement is maintained. Applicant's arguments filed 07/17/2009 have been considered but they are not persuasive.

The cited Reference is not considered.

Applicant's arguments with respect to the cited reference by James G. Speight are not considered here, as the cited reference or relevant portions thereof have not been submitted in a proper Information Disclosure Statement (see Improper Information Disclosure below).

Applicant conveys that Examiner alleges that the instant specification has no guidance as to how one would identify genes within a differentially enriched DNA pool, the expression of which contributes to biosynthetic petroleum. This is not correct. Firstly, Examiner has conveyed a key and omitted qualification in this argument which is that the instant specification does not provide guidance to identify the gene or genes of instant claims for conversion of recited substances into biosynthetic coal or biosynthetic petroleum. Secondly, the limitation of "contribute" is not recited in the instant claims and Examiner has not addressed this limitation, and does not do so now as it is not found in the instant claims.

Although, the following argument was in view of the cited reference which is not considered, Applicant generally argues that the state of the art was that petroleum analytical work was sufficiently developed at the time of the instant priority date to be able to be done without undue experimentation. For this to be considered and possibly acknowledged, Applicant should properly submit evidence supporting this for review by Examiner. Furthermore, petroleum itself is not directly at issue in the claims as it is not the result of the claimed conversion, as the claims recite biosynthetic petroleum is the result of the claimed methods, from conversion by a gene or genes capable of converting recited substances into biosynthetic petroleum. Due to lack of evidence properly provided and even considering Applicant's arguments without such evidence, the argued petroleum analytical work does not give any support for enablement of the instant claims for converting recited substances by a transfected microorganism into biosynthetic petroleum.

As Applicant has not properly provided evidence to the contrary or persuasive argument otherwise, the instant claims are not enabled and remain rejected.

#### Improper Information Disclosure

The information disclosure statement filed on 07/17/2009 within Applicant Arguments/Remarks Made in Amendment fails to comply with the provisions of 37 CFR 1.97, 1.98 and MPEP § 609 because the content requirements as discussed in MPEP § 609.04(a) are not complied with and because copies of non-patent literature have not been provided. It has been placed in the application file, but the information referred to therein has not been considered as to the merits. Applicant is advised that the date of any re-submission of any item of information contained in this information disclosure statement or the submission of any missing element(s) will be the date of submission for purposes of determining compliance with the requirements based on the time of filing the statement, including all certification requirements for statements under 37 CFR 1.97(e). See MPEP § 609.05(a).

Mark Staples  
/M. S./  
Examiner, Art Unit 1637  
07/27/2009

## The intron-containing L3 ribosomal protein gene (RPL3): sequence analysis and identification of U43 and of two novel intronic small nucleolar RNAs<sup>1</sup>

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### Abstract

Isolation and sequencing of bovine and human intron-containing L3 ribosomal protein genes are here reported. They exhibit very similar organisation, both comprising 10 exons and nine introns. A polymorphic locus, involving a 19-bp deletion, was found in intron 6 of the human gene. The frequency of the two alleles has been estimated in 200 haploid genomes. In bovine and human genes intron sequences are rather different, except for limited regions, located in corresponding positions, which show a surprisingly high degree of identity. All these regions contain conserved features defining the box C/D class of small nucleolar RNAs. Demonstration is given that U43 small nucleolar RNA is encoded within the first intron of both bovine and human genes. Single nucleotide sequences, encoding two novel species of small nucleolar RNAs (U82, U83a and U83b), are located in introns 3, 5 and 7. Their expression has been investigated and a possible role of these molecules in 2'-O-ribose methylation of rRNAs is discussed. © 2000 Elsevier Science B.V. All rights reserved.

**Keywords:** L3 ribosomal protein; Gene structure; Small nucleolar RNA; U43; U82; U83

### 1. Introduction

Ribosomes are highly conserved nucleoprotein particles, composed, in eukaryotes, of four RNA species and about 80 different proteins. Mammalian genomes usually contain multiple gene copies for each ribosomal protein, but only one of them is a functional intron-containing gene. The only known exception is represented by human S4 ribosomal protein, whose two isoforms are encoded by two active intron-containing genes, located on chromosomes X and Y, respectively [1].

Abbreviations: snoRNA, small nucleolar RNA; UTR, untranslated region; BL3, bovine L3; HL3, human L3

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<sup>1</sup> The nucleotide sequence data of BL3 and HL3 genes have been submitted to the EMBL/GenBank/DDJB nucleotide sequence databases under accession numbers AJ238851 and AJ238852; human U43 under accession number AJ238853.

In spite of the importance of these gene products in building an efficient translation machinery, gene structure is known for only a minority of them. The structure and/or the organisation of only eight genes, out of nearly 50 encoding different proteins of the large mammalian ribosomal subunit, have so far been reported, namely of genes encoding L1, L7, L7a, L9, L19, L23A, L30, L32 and L3L proteins [2–12]. The latter case refers to a L3-like ribosomal protein gene, found in the human genome and exhibiting a restricted pattern of tissue expression; the encoded protein is similar (about 77% identity) to, but distinct from, ribosomal protein L3 [12].

We previously reported the cDNA sequence of bovine L3 protein, confirming the high degree of identity (97–98%) of the encoded protein among mammals ([13–15]; H. Leffers, GenBank, accession number X73460). Identities were relatively high (62–72%), even if compared with L3 from lower organisms, such as insects, nematodes, plants, *Dictyostelium discoideum* and yeast [16–21]. This fact may be related to the involvement of L3 in peptidyltransferase activity [22] and/or in regulation of protein synthesis, as suggested by Yanagihara et al. [23].

With the exception of an incomplete description of exon–intron organisation of the mouse L3 gene, which contains at least five introns [14], available information about eukaryotic L3 gene structure concerns species very distant in evolution from mammals. Intron number increases with increasing complexity of organisms. Actually no introns have been found in the yeast gene [21], two introns have been reported in *D. discoideum* [20], three in *Caenorhabditis elegans* [18], four in *Toxocara canis* and *Arabidopsis thaliana* [17,19], and at least three in *Drosophila melanogaster* [16].

This is the first report analysing the intron-containing gene sequence of the ribosomal protein L3 (RPL3) in mammals.

Ribosomal protein genes can harbour, in their introns, one or more sequences encoding small nucleolar RNAs (snoRNAs) [24–26], which are generated during pre-mRNA processing [27–30]. Such snoRNAs are present in the cell as part of small nucleolar ribonucleoprotein particles (snoRNPs), involved in pre-rRNA processing and modification [24–26]. Two main groups of snoRNAs, recognisable on the basis of conserved sequence elements, are well char-

acterised and known as box C/D and box H/ACA snoRNAs. Their best known functions concern 2'-O-ribose methylation and site-specific pseudouridylation of rRNAs, respectively [26].

In this paper we demonstrate that box C/D snoRNA U43 expressed in humans [31] is encoded within the first intron of both bovine and human L3 genes. Moreover, we identified two novel species of snoRNAs belonging to box C/D class in introns 3, 5 and 7 of the bovine L3 gene. In the corresponding introns of the human L3 gene the same conserved structures were found. Expression of these snoRNAs was investigated and their possible role in 2'-O-ribose methylation of rRNAs is discussed.

## 2. Materials and methods

### 2.1. Materials

Taq DNA polymerase was from Sigma; Taq Dye Deoxy Terminator Cycle Sequencing kit from PE-Applied Biosystems. SuperscriptII reverse transcriptase and oligonucleotides were purchased from Gibco BRL. RNAeasy kit was from Qiagen and MAXIScript SP6/T7 kit from Ambion. [ $\alpha^{32}$ P]UTP (30 TBq/mmol) and Hybond-N filters were from Amersham Pharmacia Biotech.

Cell lines: MDBK are from bovine kidney, HeLa from human cervix carcinoma, 3T3 from mouse embryo and HepG2 from human hepatoma.

### 2.2. RNA isolation

Total RNA was extracted from beef nervous tissue and MDBK, HeLa, HepG2 and 3T3 cells, using the RNAeasy kit according to the manufacturer's instructions; nuclear RNA was extracted from MDBK cells. Nuclei were isolated according to Andersen et al. [33] with minor modifications. All procedures were carried out at 0 or 4°C using RNase-free reagents and plasticware.  $2 \times 10^7$  cells were trypsinised and washed twice in PBS, pellet was resuspended in 4 ml of RSB (10 mM Tris (pH 7.5); 10 mM NaCl; 5 mM MgCl<sub>2</sub>) containing 30% sucrose and 0.5% NP40, and centrifuged at  $400 \times g$ . Cells were resuspended with 2 ml RSB and centrifuged as described above. This step was repeated

once and the pellet was then used as starting material for RNA extraction.

### 2.3. PCR on genomic DNA and RT-PCR assays

PCRs were carried out on 20–200 ng of genomic DNA, extracted from bovine kidney and human leukocytes, or on reverse transcribed total RNA from bovine nervous tissue or nuclear RNA from MDBK cells, following standard procedures [32] in a PTC 100 (MJ Research Inc.) thermal cycler. Reaction mixtures (50  $\mu$ l) contained 1 $\times$  reaction buffer (10 mM Tris-HCl (pH 8.3); 50 mM KCl; 0.001% gelatin), 200  $\mu$ M dNTPs and 0.4  $\mu$ M primers (see Table 1). Reactions were heated at 95°C for 5 min before addition of 1.5 units of *Taq* DNA polymerase. PCR conditions were 30 s at 95°C, 30 s at 48–62°C and 0.5–5 min at 72°C, depending on the expected size of fragments, for a total of 30 cycles.

Reverse transcription was primed by random primers and carried out on 2–5  $\mu$ g of total or nuclear RNAs using 200 units of SuperscriptII reverse transcriptase following the manufacturer's instructions. RT-PCR performed on nuclear RNA was preceded by DNase I treatment.

### 2.4. PCR assays on cDNA library

PCR reactions were performed directly on 1  $\mu$ l of an undiluted bovine liver library (Clontech) using two  $\lambda$ gt10-specific primers, located in opposite directions on either side of the cloning site, i.e. CCCACCTTTTGAGCAAGTTC and ATAACAGAGGTGGCTTATGAG. Each oligonucleotide was used in separate reactions as forward primer with different L3 cDNA-specific reverse primers located in the 5' portion of the L3 coding sequence [13]. PCR reactions were carried out as above, except for a longer starting denaturation step (7 min) followed by *Taq* DNA polymerase addition. Semi-nested amplifications were performed on 1  $\mu$ l aliquots of previous PCR reactions.

### 2.5. DNA sequence analysis

In order to avoid possible errors introduced by *Taq* DNA polymerase, DNA sequencing was directly performed on PCR fragments after precipitation with

ammonium acetate, using as template 50–100 ng of the products. Sequence analysis was carried out on both strands of each DNA fragment by *Taq* dye-deoxy terminator method and an automated 370A DNA sequencer (PE-Applied Biosystems).

### 2.6. Preparation of RNA probes

DNA fragments were PCR amplified on bovine genomic DNA as described above. Suitable primer couples (U82F/U82R and U83F/U83R; see Table 1) were used to amplify a 52-bp DNA fragment of U82 and an 81-bp fragment of U83 snoRNAs. These PCR products were directly cloned into pGEM3Zl(+) vector, modified as described by Marchuk et al. [34]. Antisense RNA probes were prepared using MAXIscrip SP6/T7 kit.

### 2.7. Northern blot analysis

Northern blots of total and nuclear RNAs (20  $\mu$ g) were hybridised to [ $\alpha$ -<sup>32</sup>P]UTP-labelled U82 and U83 antisense RNA probes according to standard procedures [32]. Filter washings were carried out for 15 min each at RT in 2 $\times$ SSC (300 mM NaCl; 30 mM Na<sub>3</sub>citrate; pH 7)/0.1% SDS and at 42°C in 1 $\times$ SSC/0.1% SDS followed by 30 min at 60°C in 0.1 $\times$ SSC/0.1% SDS and 30 min at 65°C in the same solution. Filters were then exposed to Hyperfilm-MP (Amersham Pharmacia Biotech) films for 4 h at –80°C using intensifying screens.

## 3. Results

### 3.1. Isolation and sequencing of the bovine L3 gene

Among a number of primer couples assayed, primers BF1 and BR1 (Table 1) allowed the amplification of the 3' region of bovine L3 (BL3) gene. The length of the amplified fragment was 724 bp instead of 275 bp. Sequence analysis showed that this region comprised the last 275 bp of the mature transcript, interrupted by two short intervening sequences of 244 and 205 bp, respectively, likely representing the last two introns of the gene. The availability of intron sequences allowed to perform a PCR assay to isolate the entire genomic region, corresponding to the L3

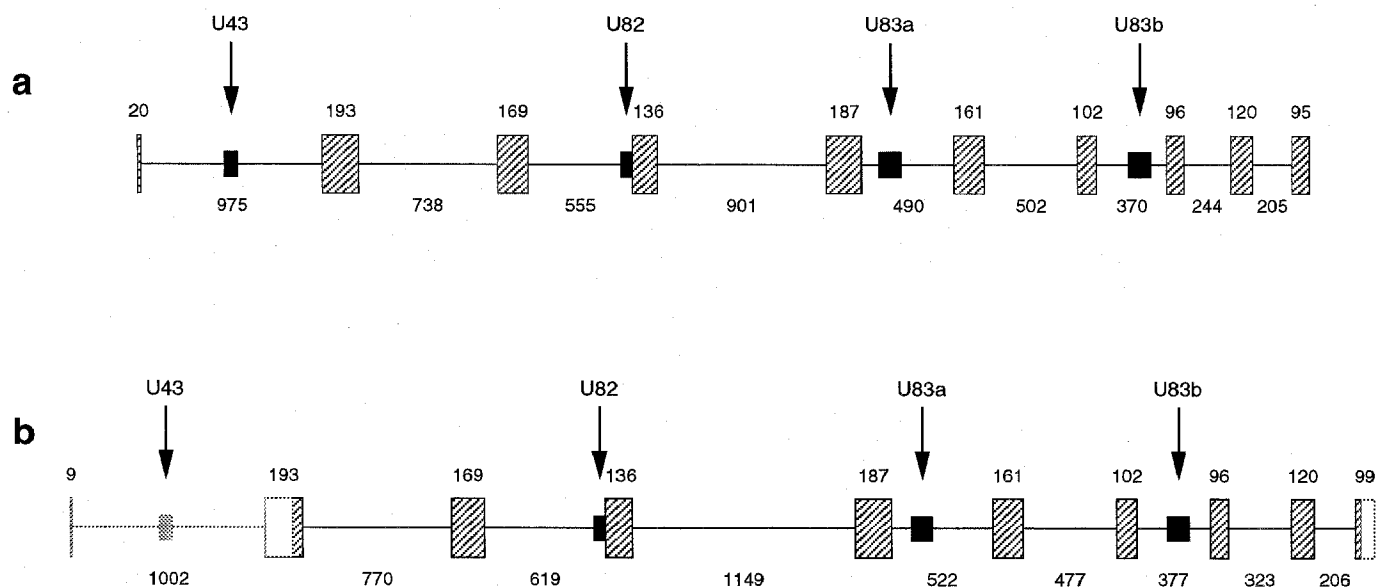


Fig. 1. Schematic outline of ribosomal protein L3 gene organisation. Comparison between bovine (a) and human (b) L3 gene structures is shown. Exons are represented by hatched rectangles, introns by lines. Locations of small nucleolar RNAs identified in the present paper are marked by black boxes. Numbers indicate base pair (bp) lengths of exons and introns. The lighter 5' and 3' ends of the human gene (b) correspond to DNA regions not isolated and sequenced by the authors.

cDNA, by the use of an intron-specific oligonucleotide (BR2) as 3' primer coupled with BF2, corresponding to the 5' end of the published L3 cDNA sequence [13]. Since no PCR products were obtained, the 5' primer (BF2) was substituted with an internal one, beginning two nucleotides downstream of the ATG start codon (BF3). This PCR product spanned about 4.6 kb and overlapped the 5' end of the above described DNA fragment of 724 bp. Sequence analysis of this 4.6-kb product allowed us to identify six additional introns.

In order to clarify the reason for the unsuccessful amplification with primer BF2, this primer was coupled with an exonic 3' primer (BR3). When tested both on genomic DNA and on total RNA extracted from nervous tissue, this couple of primers did not give any amplification product, thus excluding the presence of an intron as the cause of the failure of amplification and suggesting that the 5' UTR sequence of bovine L3 cDNA previously reported [13] might be at least partially incorrect. A re-sequencing of the original clone isolated from a bovine liver cDNA library (Clontech) confirmed the published sequence [13]. We therefore had to conclude

that this clone contained some rearrangement in the 5' region. This hypothesis was checked by performing PCR assays on aliquots of the original  $\lambda$ gt10 cDNA library. To this purpose, two 5' primers, flanking the unique *EcoRI* cloning site of the vector, were alternatively used in couple with the same downstream primer, located within the L3 coding region. These PCR reactions, followed by two semi-nested steps, the last using a 3' primer (BR4) close to the start codon, gave a main product that was subjected to sequence analysis. When compared to the published L3 sequence [13], as many as eight nucleotide substitutions were found within its 17-nt 5' UTR. A further PCR assay carried out on genomic DNA, using an upstream primer (BF4) ending at the ATG start codon of the newly isolated 5' UTR and BR4, allowed us to amplify a fragment of about 1 kb, containing the first intron of BL3 gene.

The whole sequence, resulting from the three above described overlapping fragments, spans 6259 bp and comprises 10 exons and nine introns. Apart from differences in the first exon, all the other exons showed perfect identity to bovine L3 cDNA, supporting the conclusion that the genomic sequence



Table 1  
Oligonucleotides used as primers in PCR assays

Primer	Sequence	Location	
BF1	5538 TGT CAT GCT CAA AGG CTG CGT G <sup>5559</sup>	exon	8
BF2 <sup>a</sup>	0001 GGG GCT GCC GG TAG CAT G <sup>0020</sup>	exon	1
BF3	0998 TCA CAG GAA GTT CT CTG CT CC <sup>1018</sup>	exon	2
BF4 <sup>b</sup>	0001 CAG CT TCC GAC GGC AGC AT G <sup>0020</sup>	exon	1
HF1	0001 ACA TCG TGC GGA AGT CGA <sup>0019</sup>	exon	2
HF2	1675 TCA GCAG CAT GAAGA GTAC <sup>1694</sup>	exon	4
HF3	2927 GGT GAAC GGA GGC ACT G <sup>2943</sup>	exon	5
HF4	3527 AGG AAG GGG TCA GTG GG AGG <sup>3546</sup>	intron	5
HF5	3909 G <sup>3909</sup> TAAGG TGC CAT CCG TAG <sup>3927</sup>	intron	6
BR1	6259 CACTG GAAAATAATTTTATTGAGG <sup>6236</sup>	exon	10
BR2	5765 AGGGATGAGGCAAGGATACAGATG <sup>5742</sup>	intron	8
BR3	2095 CAGTTCTTGTAGAAGCGCCTT <sup>2075</sup>	exon	3
BR4	1027 CCATGCCCTGGGAGCAGAG <sup>1010</sup>	exon	2
BR5	2690 CTTGCAGTATTTGGTGAAGG <sup>2671</sup>	exon	4
HR1	1629 TGCAGTACTTGGTAAAGGCC <sup>1610</sup>	exon	4
HR2	2932 TTCACCTGGATCTCCATCAG <sup>2913</sup>	exon	5
HR3	3625 TTGCGGGGCAGCTTCTTGG <sup>3607</sup>	exon	6
HR4	5475 TTGCAATTCGGTCTTTCTTCA <sup>5455</sup>	exon	10
HR5	4020 TACAACACAGGCCTGTAC <sup>4002</sup>	intron	6
U82F	2599 TGATGAAGGCTGGCACCCTG <sup>2617</sup>	intron	3
U82R	2650 CTT CAGGAGCAGAGCAGAGT <sup>2631</sup>	intron	3
U83F	3940 CAGTGATGAGGCCCTGGAATGA <sup>3961</sup>	intron	5
U83R	4020 TCAGAAGGAAGGCAGCAAGGAA <sup>3999</sup>	intron	5

The position of each oligonucleotide is indicated by numbers preceding and following the sequences. Numbering corresponds to L3 genomic sequences determined in this study. B, bovine; H, human; F, forward; R, reverse.

<sup>a</sup>Sequence corresponding to the 5' end of the published L3 cDNA [13].

<sup>b</sup>Sequence corresponding to the newly isolated 5' UTR of BL3 cDNA.

described here corresponds to the BL3 gene. The overall organisation of this gene together with exonic and intronic lengths are reported in Fig. 1a.

### 3.2. Analysis of bovine gene structure and identification of two novel snoRNAs

Analysis of the general gene features evidenced that splice junctions show nearly perfect identity with the consensus sequences, especially at donor splicing sites. The acceptor splicing sites are always preceded by a polypyrimidine element, uninterrupted only in introns 6 and 9.

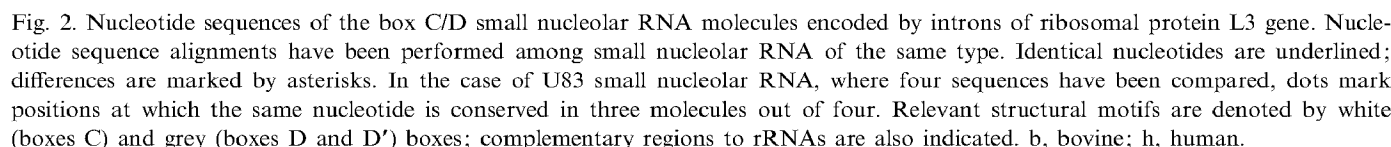
The most striking and specific features of this gene are represented by three sequences exhibiting a number of structural elements typical of a class of snoRNAs, in particular the conserved sequence elements known as boxes C (UGAUGA) close to the 5' end, and D (CUGA) close to the 3' end (Fig. 2). The first sequence is located in the middle of intron 1 (posi-

tions 484–545), the second in the terminal part of intron 3 (positions 2595–2650), and the last in introns 5 and 7 (positions 3934–4027 and 5293–5388) (Fig. 1a).

The snoRNA encoded by the first intron was found to be very similar (about 89% identity) to human U43 snoRNA [31], retaining an additional feature shared by most intron-encoded snoRNAs, i.e. complementarity to a rRNA sequence. In this case, immediately upstream of a box D' (additional box D-like motifs, present in internal locations in many snoRNAs), the same 10-nt pairing to 18S rRNA as in human U43 is present (Fig. 2).

The sequence in intron 3 exhibits, upstream of box D, 10 nt exactly matching positions 2910–2919 of 28S rRNA (according to the numbering of human 28S rRNA, GenBank accession number U13369). This sequence in intron 3 does not show any complementarity between its terminal parts.

The two sequences, in introns 5 and 7, show more



No sequences similar to these bovine snoRNAs and/or complementary to their matching 28S and 18S rRNA segments were found in a BLAST global search in the GenBank database. Two novel species of box C/D snoRNAs are therefore encoded in three introns of the BL3 gene. SnoRNAs encoded in introns 3, 5 and 7 were named, using progressive numbering, U82 and U83a and b, respectively (5' and 3' ends of these new snoRNA species have been arbitrarily assigned).

In order to verify the presence of snoRNA-like sequences in other L3 genes, human L3 (HL3) gene was amplified from genomic DNA (at that time among mammals only our bovine L3 genomic sequence was known). Four primer couples (Table 1), designed on the basis of HL3 mRNA sequence (H. Leffers, GenBank accession number X73460), were used to obtain most of the region corresponding to the primary transcript. Direct sequencing of the four PCR products showed that the first one (1629 bp), amplified using primers HF1 and HR1, contained introns 2 and 3; the second and the third ones (1258 and 699 bp; primer couples HF2/HR2 and HF3/HR3) contained one intron each, i.e. introns 4 and 5 respectively; the last one (1930 bp; primer couple HF4/HR4) contained the remaining 3' por-

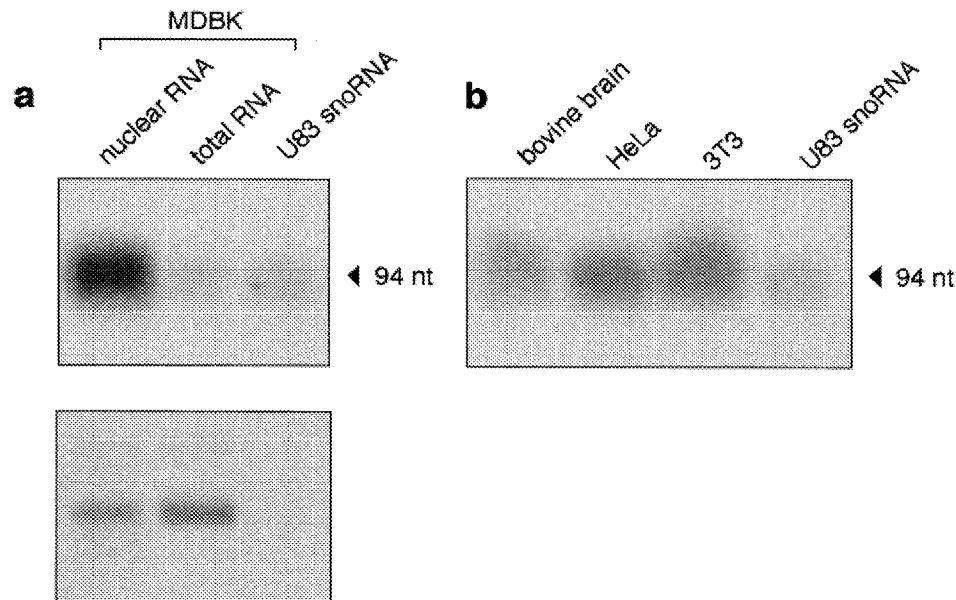


Fig. 3. Expression of U83 small nucleolar RNA evaluated by Northern blot analysis. (a) Filter in the upper part, carrying nuclear and total RNAs from a bovine cell line (MDBK), was hybridised to an [ $\alpha$ - $^{32}$ P]UTP-labelled antisense RNA probe corresponding to an 81-nt fragment of bovine U83a small nucleolar RNA; a diluted and unlabelled sense RNA fragment, identical to the bovine U83a sequence (94 nt) reported in Fig. 2, was used as RNA size marker. In the lower part of this panel, the same filter was hybridised to a mouse  $\beta$ -actin probe. (b) The same antisense RNA probe described above was used to hybridise a filter carrying total RNA from bovine brain and from human (HeLa) and mouse (3T3) cell lines. Size marker was the same as in panel a. No attempt to normalise hybridisation intensities by a  $\beta$ -actin internal control was made, owing to the expected differences in cross-hybridisation in different species.

tion of the gene, which comprised four additional introns (introns 6–9).

The presence of a possible first intron, interrupting the coding region between the start ATG and the second codon, could not be verified as only a very short sequence (6 nt) of the 5' UTR was known. At this stage of our work, the sequence of a human genomic clone of about 123 kb became available (G. Coville, GenBank accession number AL022326). This clone contained the whole L3 gene.

A comparison between the two human gene sequences evidenced some differences, all concerning untranslated regions. In particular four base substitutions were found: in our sequence A (nt 1333), C (nt 1457), A (nt 2070) and G (nt 2124) instead of G, T, G and A, respectively. By analysing the sequence of this clone, the first intron of 1002 bp just after the start codon ATG could be identified, as expected.

The whole HL3 gene spans about 6.7 kb and is composed of 10 exons and nine introns (Fig. 1b).

A search for snoRNA structures resulted in the identification of sequences carrying the canonical

motifs of box C/D snoRNAs, in introns 1, 3, 5 and 7 (Figs. 1b and 2).

The box C/D snoRNA, found in intron 1 at positions 491–552 of the L3 gene and corresponding to positions 52150–52089 of the deposited genomic clone, was identical to the already reported human U43 [31] and very similar (about 89% identity) to the corresponding bovine U43.

Intron 3 harbours at its 3' end (nt 1537–1591, according to the numbering of our deposited sequence) a sequence very similar (about 93% identity) to U82 snoRNA, above described for the bovine gene. Actually, this snoRNA represents the 3' region of a longer sequence (205 bp, nt 1387–1591), exhibiting more than 94% identity with a sequence of 206 bp, located at corresponding positions (2445–2650) in the bovine gene.

Two sequences, found at positions 3125–3219 and 4521–4613 of the gene in introns 5 and 7 respectively, showed about 80% identity. They correspond to the U83 snoRNAs identified in the bovine gene.

Intron 4 harbours a 293 bp long *Alu*-Sp-like repeat

at positions 2180–2472 of our gene sequence. In the above mentioned sequence present in GenBank, the same region spans one additional nucleotide (294 bp) since our analysis resulted in a row, at the 5' end of this repeat, of 12 instead of 13 T nucleotides.

Furthermore, during sequence analysis, a deletion of 19 bp representing a direct imperfect repeat, located in intron 6 at positions 3930–3948 (according to the numbering of our deposited sequence), was found. In order to evaluate the frequency of this 19-bp deletion in humans, a suitable DNA fragment was amplified by the use of two primers flanking the deletion site (HF5/HR5). Assays, performed on 200 human unrelated haploid genomes from a Northern Italian population, showed that the 19-bp deletion allele has a frequency of about 3%.

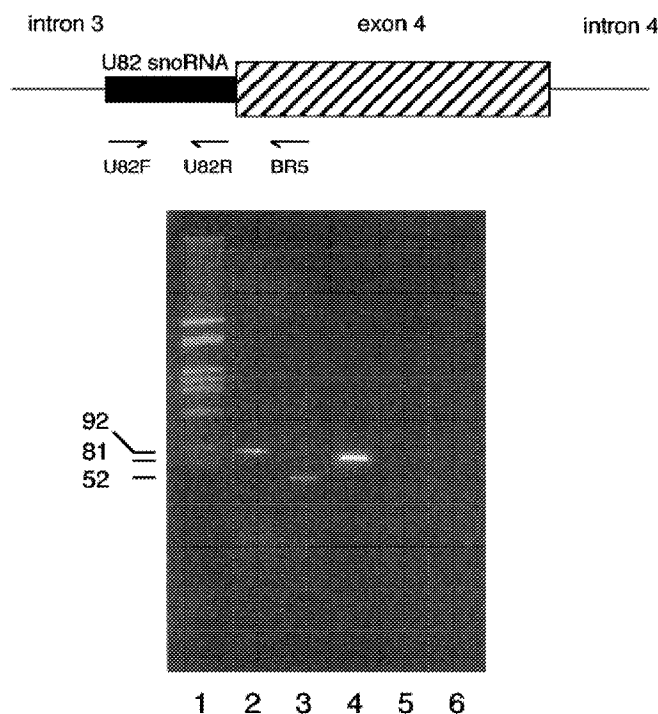


Fig. 4. RT-PCR assays on nuclear RNA from MDBK cells. Top: Schematic drawing showing the positions of primers used in assessing U82 expression. Bottom: DNase I-treated nuclear RNA was used as template to amplify different products. Amplifications were performed with the following primers: lane 2, U82F/BR5; lane 3, U82F/U82R; lane 4, U83F/U83R. Lanes 5 and 6, amplifications carried out as in lanes 2 and 3, respectively, but omitting the reverse transcription step. Lane 1, pUC8 *Hae*III.

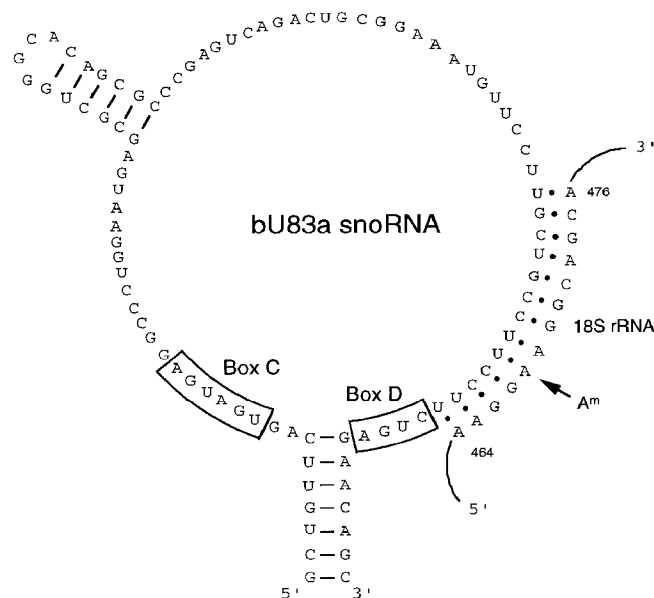


Fig. 5. Secondary structure model of bU83a small nucleolar RNA. The consensus sequences of the conserved box elements (boxes C and D) are boxed. The 13 nt region, adjacent to box D, paired to 18S rRNA is shown, together with the nucleotide ( $A^{468}$  of human 18S rRNA indicated by an arrow) that is the expected target of the 2'-O-methylation reaction.

### 3.4. Expression of U82 and U83 snoRNAs

Expression of the novel putative snoRNAs identified in both bovine and human L3 genes was estimated in bovine, human and mouse cell lines as well as in bovine brain.

Northern blot hybridisation using a bovine U83a snoRNA-specific probe allowed us to demonstrate the presence of this snoRNA in all tested samples (Fig. 3). Moreover, as expected, the radioactive signal appeared to be stronger in the case of nuclear RNA (Fig. 3a).

No hybridisation signal was detectable when the same samples were hybridised with a bovine U82 snoRNA-specific probe. Expression of U82 snoRNA was further investigated by RT-PCR. Reverse transcript obtained from DNase I-treated nuclear RNA was used as template to amplify U82 and U83 snoRNAs (the latter as positive control), using the same primers (U82F/U82R and U83F/U83R) designed for preparation of RNA probes (see Section 2 and Table 1). In order to verify the possible amplification from unprocessed heterogeneous nuclear RNA, amplification of U82 was also performed using the same up-

stream primer (U82F) and a downstream primer (BR5) located in the adjacent exon 4 (Fig. 4, top). A faint band was detectable also in this case (Fig. 4, lane 2). The absence of amplifiable genomic DNA was verified by performing RT-PCR for U82, using the primer couples U82F/BR5 and U82F/U82R, but omitting the reverse transcription step (Fig. 4, lanes 5 and 6).

#### 4. Discussion

Sequencing and structural analysis of ribosomal protein L3 genes from two mammals are here reported. It is known that the identification of ribosomal protein genes may be difficult, due to the presence of intronless pseudogenes [9]. They have been evidenced also in cattle genome both by Southern hybridisation using L3 cDNA as probe [13] and by chromosome mapping [35].

Both bovine and human L3 genes exhibit a very similar organisation. They comprise 10 exons and nine introns always interrupting the coding tract. Nucleotide sequences of all corresponding introns, in these two mammalian species, are rather different, except for some limited regions that will be discussed below.

During experimental work, some problems arose in isolating the first intron of the bovine L3 gene. The presence of rearrangements in the 5' UTR of the original L3 cDNA clone [13] did not allow us to use its information for isolating the first intron. This result was only obtained when sequence information from a newly isolated cDNA fragment was used.

As far as the first intron of human L3 gene is concerned, sequence information was derived from a large (about 123 kb) genomic clone, which was deposited in GenBank (accession number AL022326), during the final stage of this work. This clone contained the whole L3 gene, whose sequence was nearly identical to that of the nine exons and eight introns we isolated, except for four base substitutions, all concerning untranslated regions, and for one bp difference in the length of an *Alu*-Sp-like repeat in intron 4. In the human L3 gene a polymorphic locus, resulting from a 19-bp deletion in intron 6, was identified. This biallelic polymorphism

can be easily detected by PCR and may be a useful genetic marker, since the HL3 gene has been mapped to the sub-telomeric region of chromosome 22 [36].

In both genes, all splicing sites are located in exactly the same positions. The presence of the splice site for the first intron closely before or after the initiator ATG is frequent in ribosomal protein genes and has always been observed in the large subunit protein genes so far described [3–11]. This feature holds also for bovine and human L3 genes here described. In the case of the murine L3 gene too, where only a partial organisation has been reported, without any intron sequence information, the authors stressed that the first exon is “spliced precisely after the initiator methionine codon” [14].

The most interesting common features of bovine and human L3 genes are represented by some sequences, located at corresponding positions in introns 1, 3, 5 and 7, and exhibiting a surprisingly high degree of identity. In these sequences structural elements which characterise the stem-box structure of a growing family of snoRNAs, called box C/D snoRNAs, can be recognised. SnoRNAs are usually encoded by introns, which in a few instances are part of genes not encoding protein products [37,38], in most cases of protein coding genes [25–28,39,40]. The host genes of these snoRNAs, at least in vertebrates, often encode ribosome-associated or nucleolar proteins [41]. Nine different snoRNAs, all belonging to the box C/D class, encoded by introns of four mammalian ribosomal L-type protein genes, have so far been reported [2,40,42]. In addition to conserved boxes C (UGAUGA) and D (CUGA) close to the 5' and 3' ends, respectively, a further feature of most intron-encoded snoRNAs is the presence, often immediately upstream of box D (or D'), of extensive sequence complementarities (10–22 nucleotides) to sequences of the mature rRNAs, usually in the so-called universal core regions of the secondary structure [25]. It has recently been demonstrated that the box C/D motif is the intranuclear RNA trafficking signal, directing these snoRNAs from the nucleoplasm to the nucleolus [43,44].

In both L3 genes, the terminal portions (about 200 bp) of intron 3 are nearly identical (more than 94% identity), containing at the 3' end the newly identified putative snoRNA (U82, Fig. 2). In both species U82 snoRNAs exhibit the conserved boxes C and D,

but lack any base pairing between the 5' and 3' ends, a feature also found in other members of this snoRNA family, for instance in U8, U13 and U18, which at the 3' side of box D contain only two nucleotides [25,44]. Curiously, the correct 3' end of the putative U82 snoRNA could be directly generated by the splicing of the host pre-mRNA, since the splice site is located precisely two nucleotides downstream of box D. At the 5' side of this same box D, a stretch of 10 nucleotides, complementary to a sequence of 28S rRNA (positions 4976–4985, according to the numbering of human 28S rRNA, GenBank accession number U13369), perfectly conserved among vertebrates, is present. Although several typical features of box C/D snoRNAs are recognisable in the U82 sequence, the results obtained from both Northern blot and RT-PCR analyses did not allow us to demonstrate its expression. On the other hand, it should be noted that the highly conserved region of intron 3 is significantly longer than the snoRNA sequence, suggesting some other unknown role. Searches in the GenBank database did not indicate any significant similarities with other sequences. Further investigations are needed in order to verify if this conserved region holds some functional significance.

The sequences of about 100 bp, repeated in introns 5 and 7 of both bovine and human L3 genes, with about 73% identity, share common structural elements identifying a second novel snoRNA, U83 (Fig. 2). These sequences perfectly fit the 'terminal core motif', defined by Xia et al. [45], consisting of a base-paired 5', 3'-terminal stem with flanking box C and D consensus sequences (Fig. 5). This motif also comprises two unpaired nucleotides, always separating the terminal stem from box C. Removal of these two unpaired nucleotides from U14 abolished its processing [45]. Moreover, a sequence pairing with 18S rRNA at positions 464–476 (according to the numbering of human 18S rRNA, GenBank accession number U13369) is located immediately upstream of box D. This complementarity region spans 13 nt of perfect matching in the bovine snoRNAs, while it shows one mismatch close to its 5' end (after 11 or 10 continuous pairings) in the human U83a and b snoRNAs. Unfortunately nucleotide sequences of both 18S and 28S rRNAs are unknown in cattle, nevertheless the sequences complementary to U82 and U83 snoRNAs fall within highly conserved re-

gions among 28S rRNAs from higher eukaryotes and among 18S rRNAs from both higher and lower eukaryotes [46].

Expression of U83 snoRNA has been evidenced by Northern blot analysis, not only in bovine and human, but also in mouse cells. This suggests that U83 snoRNA is normally expressed at least in mammalian tissues. The high level of identity, among the four U83 snoRNAs here characterised, did not allow us to establish if both of them or only one member of the couple present within the same gene are actually expressed.

Among several roles that can be argued for snoRNAs, such as chaperones in rRNA folding or as molecules involved in assembly of rRNP complexes, their currently best known function concerns rRNAs processing [24,31]. In particular, box C/D snoRNAs are involved in 2'-O-ribose methylation of pre-rRNAs. The antisense element plays a direct role through base-pairing interaction, thus providing the information to select the target nucleotide (always the nucleotide paired to the fifth one upstream of box D) for the modification reaction [25,26,31,40].

According to this rule, in the case of the novel snoRNA U83, the target nucleotide within 18S rRNA would be adenylate 468, which has already been reported to be 2'-O-ribose-methylated in eukaryotic 18S rRNA [31,47,48]. This observation agrees with and gives further support to the U83 expression data here presented. It is interesting to note that the complementary region of 18S rRNA consistently overlaps (eight nucleotides) the sequence matching U14 snoRNA immediately upstream of its box D [31]. Actually, U14 snoRNA selects the target nucleotide for methylation six positions downstream in the 18S rRNA sequence [31,49]. Overlapping sequence complementarities to U32 and U33 in 18S rRNA, as well as to U18 and U21 snoRNAs in 28S rRNA, have been reported [25,31,40,42].

Moreover, a sequence corresponding to U43 [31] was found within intron 1 of both genes (Fig. 2). Since the origin of this molecule was unknown, we demonstrated that it is encoded by the first intron of mammalian L3 genes.

The known intronic sequences of the other described L3 genes (*C. elegans*, *A. thaliana* and *D. discoideum*) [18–20] do not harbour any sequence similar to snoRNAs. This is not surprising, because "a

specific snoRNA can be encoded in different parent genes in different organisms, and the number of snoRNA genes in the parent gene and intron location are not strictly conserved” [24]. Curiously, U14 snoRNA is intron-encoded in mammals, amphibians and fishes, but it is non-intronic in *S. cerevisiae*, while it is encoded by tandemly arranged genes in maize ([24] and references therein).

In conclusion, the high degree of structure conservation of limited regions, within the bovine and human sequences here described, supports the hypothesis that introns of mammalian L3 genes encode the same snoRNAs. Thus, as suggested by Qu et al. [50] for host genes with multiple single-snoRNA-containing introns, the mammalian L3 gene constitutes a snoRNA polycistronic unit fused to the protein coding sequence.

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## Review

## What is a gene? An updated operational definition

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## ABSTRACT

A crucial pre-requisite for large-scale annotation of eukaryotic genomes is the definition of what constitutes a gene. This issue is addressed here in the light of novel and surprising gene features that have recently emerged from large-scale genomic and transcriptomic analyses. The updated operational definition proposed here is: "a gene is a discrete genomic region whose transcription is regulated by one or more promoters and distal regulatory elements and which contains the information for the synthesis of functional proteins or non-coding RNAs, related by the sharing of a portion of genetic information at the level of the ultimate products (proteins or RNAs)". This definition is specifically designed for eukaryotic chromosomal genes and emphasizes the commonality of the genetic material that gives rise to final, functional products (ncRNAs or proteins) derived from a single gene. It may be useful in several applications and should help in the provision of a comprehensive inventory of the genes of a given organism, finally allowing answers to the basic question of "how many genes" are encoded in its genome.

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## 1. Problematic issues in eukaryotic chromosomal gene definition

A major goal of a genome sequencing project for a specific organism is the definition of its entire gene complement. To accomplish this important task several fairly accurate gene prediction tools are generally used together with large-scale production of expression evidence (e.g. cDNA and EST sequences).

In this review I will deal with the problem of the definition of what is a gene, a crucial pre-requisite for large-scale annotation of eukaryotic genomes. Indeed, gene assessment in prokaryotic genomes is much simpler owing to their higher gene density (about 80% of a prokaryotic genome is protein coding) and the lack of introns. The

identification of significantly large Open Reading Frames (ORFs) is an obvious solution for the identification of the majority of protein coding prokaryotic genes. Short prokaryotic genes are more problematic but can generally be identified with suitable bioinformatics approaches validated by transcription and translation evidence.

To date, 77 eukaryotic genome projects have been completed (Liolios et al., 2006) but for none of them are we able to answer the simple question of how many genes they contain. This is mostly due to the presence of some gaps in the genome sequences and to the incompleteness of gene annotation. However, even if all gaps were closed and a full gene annotation was available and validated by comprehensive transcriptional evidence, we could be still unable to provide reliable estimate of the gene number – in part because of the lack of a clear and unambiguous definition of what a gene is.

Several definitions have been proposed – such as this, from one of the most widely used Molecular Biology textbooks: "A gene is the segment of DNA specifying a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding

Abbreviations: AS, alternative splicing; miRNA, micro RNA; ncRNA, non-coding RNA; ORF, open reading frame; TSS, transcription start site; TTS, transcription termination site; TU, transcriptional unit; UTR, untranslated region.

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segments (exons)." ((Lewin, 2007), <http://www.ergito.com/>). This exemplar definition, apart from its ambiguous use of the term "exon", is barely satisfactory as it does not consider some problematic gene features recently highlighted by work carried out at the RIKEN Institute on the transcriptional landscape of mouse genome (Carninci et al., 2005) and most recently by the International Encyclopedia of DNA elements (ENCODE) project (Gerstein et al., 2007) that strongly challenge the conventional view of genes. Indeed, the classical "one gene – one protein" definition is no longer acceptable and is also impractical (Pearson, 2006).

In particular:

- 1) A large fraction of genes do not encode for proteins. Indeed, over 50% of the transcriptional units (TUs) identified in mouse do not appear to be coding and the majority of them are alternatively spliced and polyadenylated.
- 2) The same gene locus may encode a large variety of transcripts and proteins through alternative transcription start sites (TSS), alternative transcription termination sites (TTS) and alternative splicing (AS). In some cases AS may generate mRNAs encoding for completely unrelated proteins using different coding frames.
- 3) Some genes have been found to overlap each other on the same or opposite strands. The discontinuous structure of eukaryotic genes potentially allows "Russian doll" gene models, where one gene can be completely contained inside one or more introns of another gene without sharing any exonic regions.
- 4) The ligation of two distinct mRNA molecules encoded by separate gene loci through the *trans*-splicing mechanism is another phenomenon widespread in some eukaryote lineages such as

nematodes and ascidians (Hastings, 2005) which may further increase the complexity of the gene expression pattern.

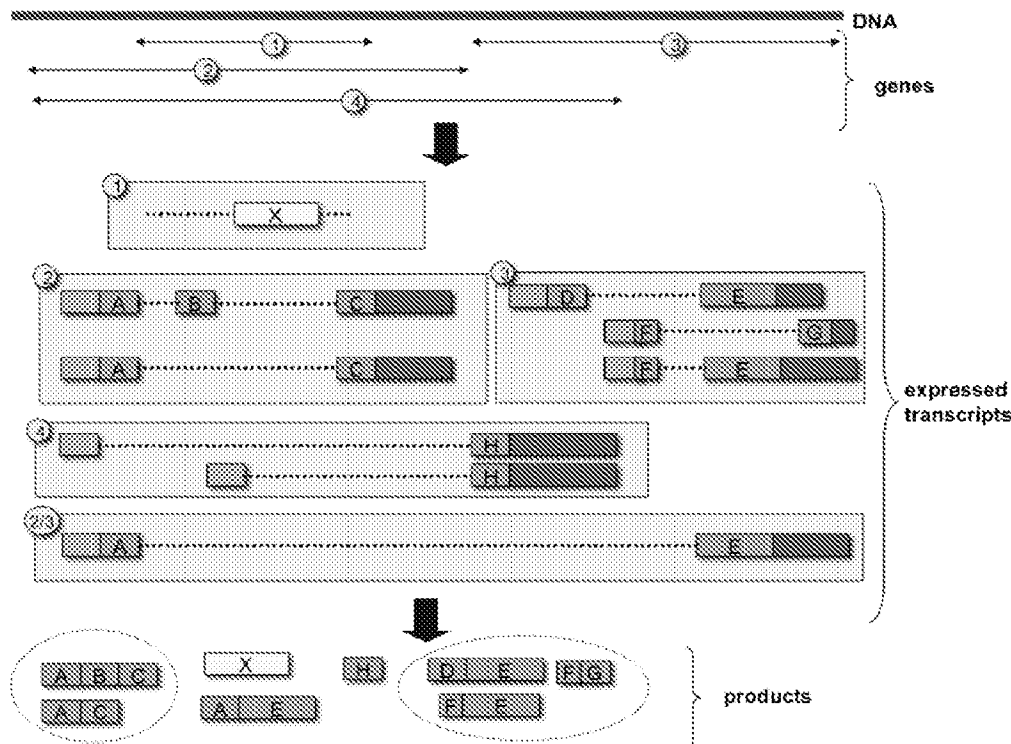
- 5) Finally, recent computational and experimental analyses point to the existence of chimerical transcripts produced by the co-transcription of tandem gene pairs, and potentially encoding fusion proteins (Parra et al., 2006).

## 2. An updated operational gene definition

In the light of the above features one might ask if it is still appropriate to maintain a gene-centric view of molecular biology, or it is better to just consider functional products (proteins and ncRNAs) that may be in some way related by the molecular processes involved in their expression, such as the sharing of a promoter (or TSS), a transcriptional termination (TTS) or one or more splicing sites. Indeed, to understand the relationships between the different cellular components in a system biology framework, it may be more appropriate to consider functional products rather than genes, in the light of their specific expression in different conditions (i.e. tissue, developmental stage or pathological status).

However, I believe that despite the many problems that have emerged in these last years it would be premature to announce the death of the gene concept, mostly because the tight connection between a functional product and its encoding genetic material cannot be disregarded. However, an updated operational definition is needed to allow the unambiguous association between transcripts, proteins, and their encoding genes.

In agreement with Gerstein et al. (2007) this updated definition should adopt a bottom-up criterion, i.e. emphasize the ultimate



**Fig. 1.** The discrete genomic region depicted here encodes one non-coding and eight protein coding spliced transcripts (ncRNA in yellow; 5'UTR and 3'UTR in light and dark pink, respectively; protein coding sequence in green; dotted lines represent RNA removed or spliced out by maturation). Four different genes (numbered 1–4) can be annotated according to the gene definition proposed here. A specific set of transcripts can be clustered and assigned to the same gene if the transcript projections on the genome sequence – limited at the regions encoding the final products (e.g. the green and the yellow boxes for the protein coding and non-coding RNA genes, respectively) – overlap each other. The clustering procedure is iterated and may include in the same gene cluster non-overlapping transcripts. For example, in the case of gene 3, the transcript isoforms encoding for products DE and FE are clustered because they overlap through the region E, then the transcript FG is added to this cluster because of the overlapping of the region F with one member of the cluster. The transcript encoding the product AE can be identified as a chimerical transcript originated by the concatenation of two exons belonging to two different genes as these two exons are prevalently expressed by two unrelated genes (i.e. genes 2 and 3). The gene coordinates, denoted by the arrowed lines, are the leftmost and rightmost mapping positions on the genome of all transcripts belonging to the same gene cluster. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

functional gene products, either ncRNAs (e.g. miRNA) or proteins, and consider the regulatory regions involved in their expression at both the transcriptional (i.e. promoter, enhancer, etc.) and post-transcriptional (i.e. 5'UTR and 3'UTR) level as “gene-related”. Thus, the proposed operational definition can be summarized as: “a gene is a discrete genomic region whose transcription is regulated by one or more promoters and distal regulatory elements and which contains the information for the synthesis of functional proteins or non-coding RNAs, related by the sharing of a portion of genetic information at the level of the ultimate products (proteins or RNAs)”. This definition does not include *cis*-regulatory regions as sequence elements controlling the expression of a gene are not necessarily located upstream of it and may be dispersed throughout the genome (Gerstein et al., 2007) making the accurate definition of their boundaries unfeasible. In addition, some of the transcriptional regulatory elements may themselves be transcribed (Zhu et al., 2007).

An example to illustrate the application of this definition is shown in Fig. 1, where a genomic region encoding nine different transcripts – which give rise to one ncRNA and seven functional proteins – is described. According to the above definition: i) ABC, AC and ii) DE, FE, FG, form two clusters of related proteins, generated by alternatively spliced products of genes 2 and 3. I would suggest that two (or more) proteins are related (i.e. belong to the same gene cluster) if their encoding genome sequences overlap each other. Indeed, products with overlapping encoding genome sequences, like DE and FE, have a strict genetic relationship as a mutation in the shared genomic region

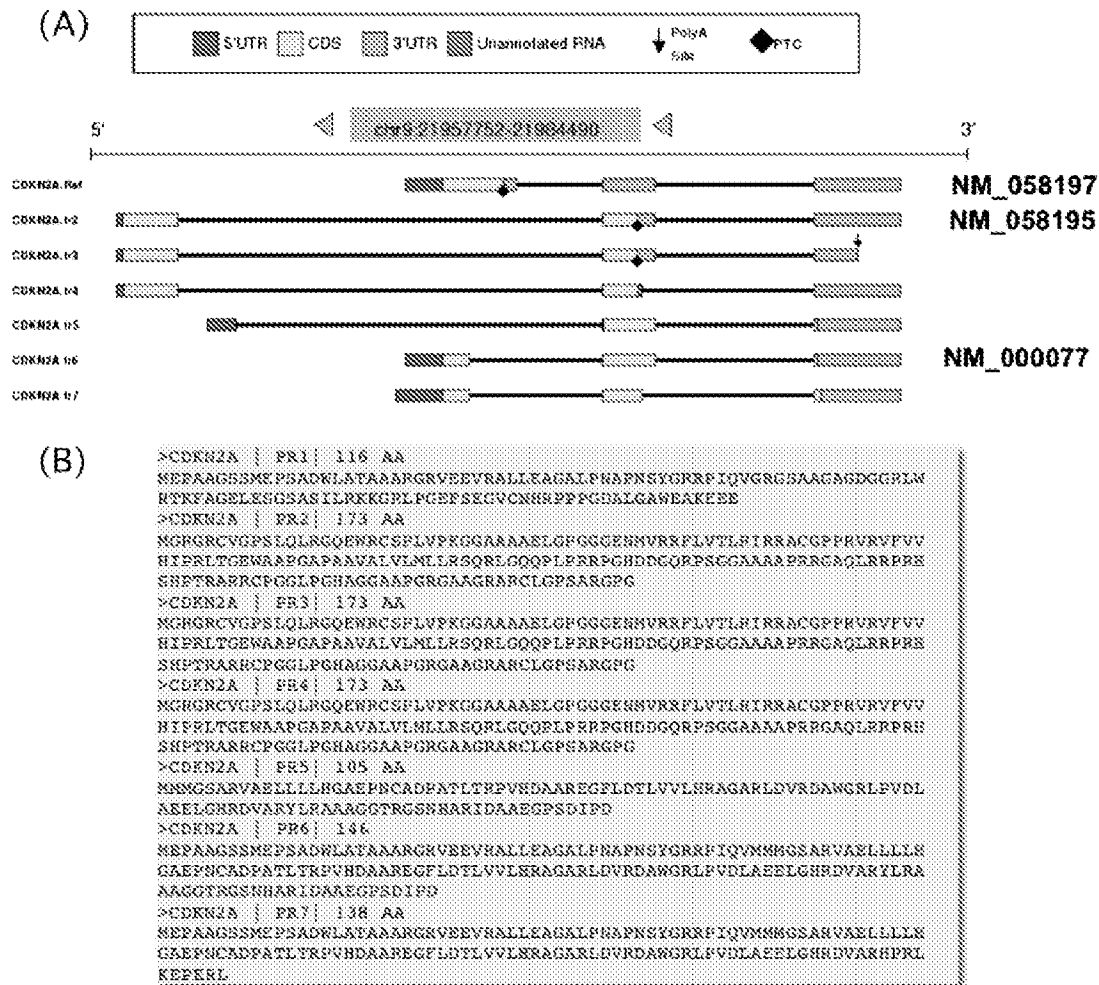
(i.e. the E region) would affect both products. It should be noted that the relationships between two products can be indirect as DE and FG are related through FE (see also the legend of Fig. 1).

Related proteins may also have completely different sequences, as in the case of DE and FG, or if the expressed products should use a different reading frame.

According to the gene definition proposed here the transcript encoding the product H should be assigned to a different gene (4), even if it shares the same TSS with transcripts encoding ABC and AC, given that H and ABC (or AC) are completely unrelated proteins, i.e. encoded by non-overlapping genomic regions. This is in line with the recent observation that different genes may share distal 5'UTRs, possibly providing a specific expression pattern (Denoeud et al., 2007). Furthermore, the existence of *trans*-splicing – where exons from two separate transcripts are spliced together to form a mature mRNA molecule – has been shown in some eukaryotes (Hastings, 2005).

In the genomic region drawn in Fig. 1, we are also able to identify an additional gene (1) encoding a ncRNA giving rise to the mature product X. This situation accounts for miRNA genes, often expressed as polycistronic primary-miRNA and located in the introns of coding or non-coding RNAs (Kim and Nam, 2006).

Finally, AE can be identified as a fusion protein originating from the co-transcription of two tandem genes (2 and 3, expressing non-overlapping mature transcripts) through the formation of a chimerical transcript – on the basis that the prevalent expression forms of the genes which provide exons to this product form two unrelated transcript



**Fig. 2.** (A) Seven alternative mRNAs expressed by CDKN2A gene in human as determined by the ASPIC program (Castrignano et al., 2006) (RefSeq IDs are shown on the right of known isoforms). (B) Alternative proteins encoded by transcript isoforms shown in (A).

clusters, i.e. with the 3' end of the transcripts of the first cluster lying upstream of the 5' end of the transcripts of the second cluster, and encode unrelated and non-overlapping functional products.

Once the related mature products have been defined one can easily go back to the relevant precursor transcripts, and determine the gene coordinates on the genome as their leftmost and rightmost mapping positions (Fig. 1). In this way a single gene locus is defined to encode a set of "related" products and its genomic coordinates established by precursor transcripts.

The gene definition proposed here is different from the one proposed by Gerstein et al. (2007): "A gene is a union of genomic sequences encoding a coherent set of potentially overlapping functional products" in that in the current proposal: i) each gene is assigned a contiguous genomic region; ii) gene coordinates include 5' and 3' mRNA untranslated (UTR) sequences included in the precursor transcript. Therefore, according to the proposed definition a genomic tract encoding for a *trans*-spliced leader is not included in the genomic region assigned to a given gene as we assume that a gene is "a contiguous genome region" and furthermore the *trans*-leader corresponds to an "untranslated" region of the transcript which do not contribute to the final product.

The definition provided in the current paper is not only simpler but also operationally more appropriate as it unambiguously defines the genomic region to be considered in the analysis of alternative splicing – usually carried out by aligning gene-related transcripts (typically a Unigene cluster) to the relevant genomic region where alternatively spliced 5'UTRs are frequently observed.

To deal with a real example, Fig. 2 shows the splicing pattern of the gene CDKN2A, as determined by the ASPIC program (Castrignano et al., 2006). It should be noted that the first and second transcripts (CDKN2A.Ref and CDKN2A.Tr2 in Fig. 2A) encode two completely different proteins, 116 and 173 aa long respectively (Fig. 2B) and the corresponding coding sequences use different reading frames. CDKN2A.Tr2, .Tr3 and .Tr4 encode the same product but differ in their 3'UTR. CDKN2A.Tr5, .Tr6 and .Tr7 encode different partially overlapping proteins of 105, 146 and 138 residues, respectively. Note that products of CDKN2A.Ref and CDKN2A.Tr5 are indirectly related through the product of CDKN2A.Tr6.

This example highlights a possible problem that may arise with the proposed definition. Indeed, in most real gene predictions we know neither the location of the coding sequence, if any, nor the function of the encoded protein. In fact, in this case only CDKN2A.Ref, .Tr2 and .Tr6 correspond to known transcripts included in the RefSeq collection (Pruitt et al., 2007). A pragmatic solution to this problem is to annotate the longest possible open reading frame as a functional product (even in the absence of strong supporting data). In this way all inferred transcripts, CDKN2A.Tr1–.Tr7, will be assigned to the same gene locus.

It is now quite clear that an unequivocal and universal gene definition is not possible and therefore it has been proposed that the operational units of a genome could be better represented by the different expressed transcripts as they actually relate the genome sequence to function and phenotype (Gingeras, 2007). However, the gene concept, with suitable revision and update still remains a key issue in Molecular Biology, underlying the centrality of the relation-

ship between genotype and phenotype. An operational definition, such as that proposed here may be extremely useful for the unambiguous classification of transcripts in discrete gene loci, such as those provided by the Unigene database (Wheeler et al., 2007) and may be more appropriate for computational analysis involving alignment of genome and transcript sequences. By way of contrast, the Gerstein et al. (2007) gene definition, which includes a discontinuous genome region with the exclusion of UTRs, cannot be used to delineate the genome region to be considered in bioinformatics analyses for the detection of novel splicing isoforms and of splicing events located in non-coding portion of mRNAs.

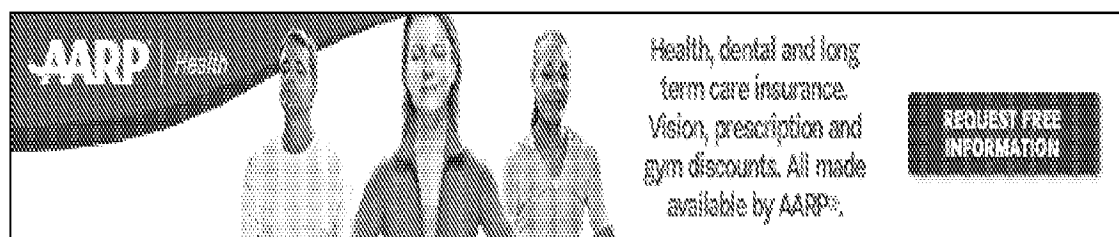
The simple operational gene definition proposed here, while not universal – it is specifically designed for chromosomal eukaryotic genes (e.g. genes of RNA viruses do not fit this definition) – allows unambiguous definition of gene coordinates and of gene-related transcripts. It may have a wide range of applicability and help in the provision of a comprehensive inventory of the genes of a given organism, finally allowing answers to the basic question of "how many genes" are encoded in its genome.

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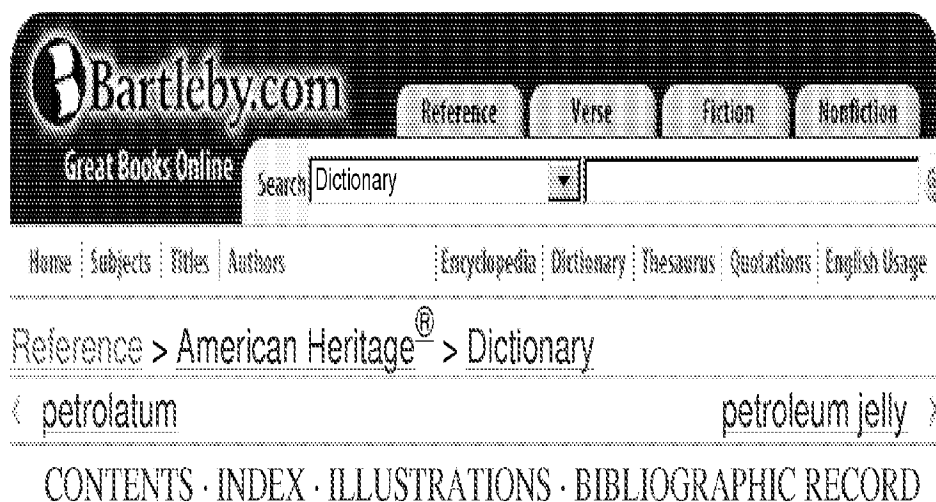
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# petroleum

SYLLABICATION: pe·tro·le·um

PRONUNCIATION:  pə-ˈtrɒl-ē-əm

**NOUN:** A thick, flammable, yellow-to-black mixture of gaseous, liquid, and solid hydrocarbons that occurs naturally beneath the earth's surface, can be separated into fractions including natural gas, gasoline, naphtha, kerosene, fuel and lubricating oils, paraffin wax, and asphalt and is used as raw material for a wide variety of derivative

products.

ETYMOLOGY: Middle English, from Medieval Latin *petr̄leum* : Latin *petra*, rock; see *petrous* + Latin *̄leum*, oil; see oil.

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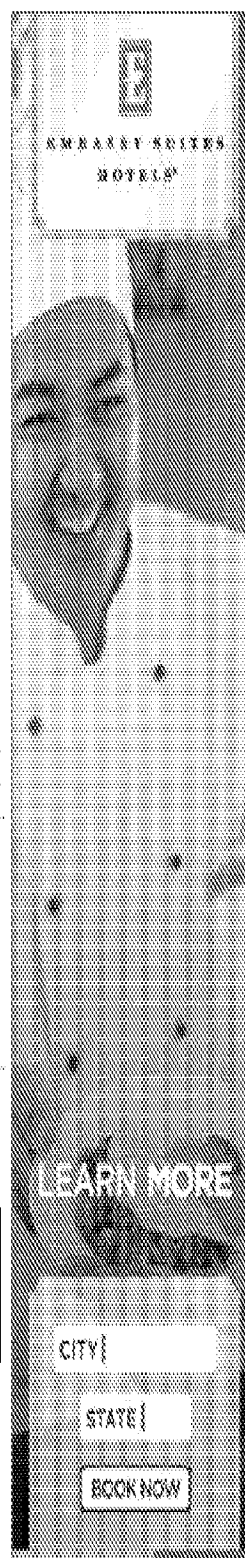
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## coal

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Photography



Location of the most-important coal occurrences on Earth.  
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solid, usually brown or black, carbon-rich material that most often occurs in stratified sedimentary deposits. It is one of the most important of the primary fossil fuels.

Noted coal geologist James Morton Schopf defined coal as containing more than 50 percent by weight (or 70 percent by volume) carbonaceous matter produced by the compaction and induration of altered plant remains—namely, peat deposits. Different varieties of coal arise because of differences in the kinds of plant material (coal type), degree of coalification (coal rank), and range of impurities (coal grade). Although most coals occur in stratified sedimentary deposits, the deposits may later be subjected to elevated temperatures and pressures caused by igneous intrusions or deformation during orogenesis (i.e., processes of mountain building), resulting in the development of anthracite and even graphite. Although the concentration of carbon in the Earth's crust does not exceed 0.1 percent by weight, it is indispensable to life and constitutes humankind's main source of energy.

This article considers the geological origins, structure, and properties of coal, its usage throughout human history, and current world distribution. For a discussion of the coal-extraction process, *see* the article coal mining. For a more complete treatment of the processes involved in coal combustion, *see* the article coal utilization.

### History of the use of coal

#### In ancient times

The discovery of the use of fire helped to distinguish humans from other animals. Early fuels were primarily wood (and charcoal derived from it), straw, and dried dung. References to the early uses of coal are meagre. Aristotle referred to “bodies which have

substances.” (It should be noted that biblical references to coal are to charcoal rather than to the rock, coal.) Coal was used commercially by the Chinese long before it was utilized in Europe. Although no authentic record is available, coal from the Fushun mine in northeastern China may have been employed to smelt copper as early as 1000 BC. Stones used as fuel were said to have been produced in China during the Han dynasty (206 BC-AD 220).

### **In Europe**

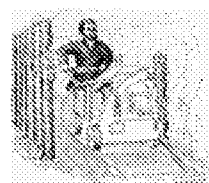
Coal cinders found among Roman ruins in England suggest that the Romans were familiar with its use before AD 400. The first documented proof that coal was mined in Europe was provided by the monk Reinier of Liège, who wrote (about 1200) of black earth very similar to charcoal used by metalworkers. Many references to coal mining in England, Scotland, and the European continent began to appear in the writings of the 13th century. Coal was, however, used only on a limited scale until the early 18th century, when Abraham Darby of England and others developed methods of using coke made from coal in blast furnaces and forges. Successive metallurgical and engineering developments—most notably the invention of the coal-burning steam engine by James Watt—engendered an almost insatiable demand for coal.

### **In the New World**

Up to the time of the American Revolution, most coal used in the American colonies came from England or Nova Scotia. Wartime shortages and the needs of the munitions manufacturers, however, spurred small American coal-mining operations such as those in Virginia on the James River near Richmond. By the early 1830s mining companies had emerged along the Ohio, Illinois, and Mississippi rivers and in the Appalachian region. As in European countries, the introduction of the steam locomotive gave the American coal industry a tremendous impetus. Continued expansion of industrial activity in the United States and in Europe further promoted the use of coal.

### **Modern utilization**

#### **Coal as an energy source**



Rail-mounted coal-cutting machine, 19th

Coal is an abundant natural resource that can be used as a source of energy, as a chemical feedstock from which numerous synthetic compounds (e.g., dyes, oils, waxes, pharmaceuticals, and pesticides) can be derived, and in the production of coke for metallurgical



*Photos.com/ Jupiterimages* production of electrical power using steam generation. In addition, gasification and liquefaction produce gaseous and liquid fuels that can be easily transported (e.g., by pipeline) and conveniently stored in tanks.

### **Conversion**

In general, coal can be considered a hydrogen-deficient hydrocarbon with a hydrogen-to-carbon ratio near 0.8, as compared with a liquid hydrocarbons ratio near 2 and a gaseous hydrocarbons ratio near 4. For this reason, any process used to convert coal to alternative fuels must add hydrogen (either directly or in the form of water). Gasification refers to the conversion of coal to a mixture of gases, including carbon monoxide, hydrogen, methane, and other hydrocarbons, depending on the conditions involved. Gasification may be accomplished either in situ or in processing plants. In situ gasification is accomplished by controlled, incomplete burning of a coal bed underground while adding air and steam. The gases are withdrawn and may be burned to produce heat or generate electricity, or they may be used as synthesis gas in indirect liquefaction or the production of chemicals.

Liquefaction may be either direct or indirect (i.e., by using the gaseous products obtained by breaking down the chemical structure of coal). Four general methods are used for liquefaction: (1) pyrolysis and hydrocarbonization (coal is heated in the absence of air or in a stream of hydrogen), (2) solvent extraction (coal hydrocarbons are selectively dissolved and hydrogen is added to produce the desired liquids), (3) catalytic liquefaction (hydrogenation takes place in the presence of a catalyst—for example, zinc chloride), and (4) indirect liquefaction (carbon monoxide and hydrogen are combined in the presence of a catalyst).

### **Problems associated with the use of coal**

#### **Hazards of mining and preparation**

Coal is abundant; assuming current rates of usage and production do not change, estimates of reserves indicate that enough coal remains to last more than 200 years. There are, however, a variety of problems associated with the use of coal.

Mining operations are hazardous. Each year hundreds of coal miners lose their lives or are seriously injured. Major mine hazards include roof falls, rock bursts, and fires and explosions. The latter result when flammable gases (such as methane) trapped in the coal are

prior to mining is expected to lead to safer mines and provide a source of natural gas that has been wasted for so long. Also, the repeated inhalation of coal dust over extended periods of time can result in serious health problems—for example, black lung.

Coal mines and coal-preparation plants have caused much environmental damage. Surface areas exposed during mining, as well as coal and rock waste (which were often dumped indiscriminately), weathered rapidly, producing abundant sediment and soluble chemical products such as sulfuric acid and iron sulfates. Nearby streams became clogged with sediment, iron oxides stained rocks, and “acid mine drainage” caused marked reductions in the numbers of plants and animals living in the vicinity. Potentially toxic elements, leached from the exposed coal and adjacent rocks, were released into the environment. Since the 1970s, stricter laws have significantly reduced the environmental damage caused by coal mining in developed countries, though more-severe damage continues to occur in many developing countries.

#### **Hazards of utilization**

Coal utilization can cause problems. During the incomplete burning or conversion of coal, many compounds are produced, some of which are carcinogenic. The burning of coal also produces sulfur and nitrogen oxides that react with atmospheric moisture to produce sulfuric and nitric acids—so-called acid rain. In addition, it produces particulate matter (fly ash) that can be transported by winds for many hundreds of kilometres and solids (bottom ash and slag) that must be disposed of. Trace elements originally present in the coal may escape as volatiles (e.g., chlorine and mercury) or be concentrated in the ash (e.g., arsenic and barium). Some of these pollutants can be trapped by using such devices as electrostatic precipitators, baghouses, and scrubbers. Current research on alternative means for combustion (e.g., fluidized bed combustion, magnetohydrodynamics, and low nitrogen dioxide burners) is expected to provide efficient and environmentally attractive methods for extracting energy from coal. Regardless of the means used for combustion, acceptable ways of disposing of the waste products have to be found.

The burning of all fossil fuels (oil and natural gas included) releases large quantities of carbon dioxide (CO<sub>2</sub>) into the atmosphere. The CO<sub>2</sub> molecules allow the shorter-wavelength rays from the Sun to enter the atmosphere and strike the Earth's surface, but they do not allow much of the long-wave radiation reradiated from the surface to escape into space. The CO<sub>2</sub> absorbs this upward-

downward, causing the lower atmosphere to remain warmer than it would otherwise be. Whereas the greenhouse effect is a naturally occurring process, its enhancement due to increased release of greenhouse gases (CO<sub>2</sub> and other gases, such as methane and ozone) is called global warming. According to the Intergovernmental Panel on Climate Change (IPCC), there is substantial evidence that higher concentrations of CO<sub>2</sub> and other greenhouse gases have increased the mean temperature of the Earth since 1950. This increase is probably the cause of noticeable reductions in snow cover and sea ice extent in the Northern Hemisphere. In addition, a worldwide increase in sea level and a decrease in mountain glacier extent have been documented. Technologies being considered to reduce carbon dioxide levels include biological fixation, cryogenic recovery, disposal in the oceans and aquifers, and conversion to methanol.

### **Origin of coal**

#### **Coal-forming materials**

##### **Plant matter**

It is generally accepted that most coals formed from plants that grew in and adjacent to swamps in warm, humid regions. Material derived from these plants accumulated in low-lying areas that remained wet most of the time and was converted to peat through the activity of microorganisms. (It should be noted that peat can occur in temperate regions [e.g., Ireland and the state of Michigan in the United States] and even in subarctic regions [e.g., the Scandinavian countries].) Under certain conditions this organic material continued to accumulate and was later converted into coal. Much of the plant matter that accumulates on the surface of the Earth is never converted to peat or to coal, because it is removed by fire or organic decomposition. Hence, the vast coal deposits found in ancient rocks must represent periods during which several favourable biological and physical processes occurred at the same time.

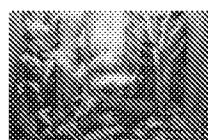
Evidence that coal was derived from plants comes from three principal sources. First, lignites, the lowest coal rank, often contain recognizable plant remains. Second, sedimentary rock layers above, below, and adjacent to coal seams contain plant fossils in the form of impressions and carbonized films (e.g., leaves and stems) and casts of larger parts such as roots, branches, and trunks. Third, even coals of advanced rank may reveal the presence of precursor plant material. When examined microscopically in thin sections or polished blocks, cell walls, cuticles (the outer wall of leaves), spores, and other structures can still be recognized (see

(Algae are major components in boghead coal, a type of sapropelic coal.)

### The fossil record

Anthracite (the highest coal rank) material, which appears to have been derived from algae, is known from the Proterozoic Eon of Precambrian time (approximately 540 million to 2.5 billion years ago). Siliceous rocks of the same age contain fossil algae and fungi. These early plants were primarily protists (solitary or aggregate unicellular organisms that include yellow-green algae, golden-brown algae, and diatoms) that lived in aqueous environments. It was not until the Late Silurian Period (approximately 420 million years ago) that plants are known to have developed the ability to survive on land. Fossil organisms that are reflective of this dramatic evolutionary event have been discovered in Wales and Australia.

Evidence for early coastal forests is preserved in strata of the Late Devonian Period (approximately 360 to 385 million years old). By the latter half of the Paleozoic Era, plants had undergone extensive evolution and occupied many previously vacant environments (this phenomenon is sometimes called adaptive radiation).



Pennsylvanian coal

forest diorama

Courtesy of the  
Department Library  
Services, American  
Museum of Natural

History, neg. #333983

There were two major eras of coal formation in geologic history. The older includes the Carboniferous Period (sometimes divided into the Mississippian and Pennsylvanian periods, from approximately 300 to 360 million years ago) and the Permian Period (from approximately 250 to 300 million years ago). Much of the bituminous coal of eastern North America and Europe is Carboniferous in age. Most coals in Siberia, eastern Asia, and Australia are of Permian origin. The younger era began about 135 million years ago during the Cretaceous Period and reached its peak approximately 2 to 65 million years ago, during the Paleogene and Neogene periods of the Cenozoic Era. Most of the coals that formed during this second era are lignites and subbituminous (or brown) coals. These are widespread in such areas as western North America (including Alaska), southern France and central Europe, Japan, and Indonesia.

Late Paleozoic flora included sphenopsids, lycopsids, pteropsids, and the Cordaitales. The sphenopsid *Calamites* grew as trees in swamps. *Calamites* had long, jointed stems with sparse foliage. The lycopsids included species of *Lepidodendron* and *Sigillaria* (up to 30 metres [about 100 feet] tall) that grew in somewhat drier areas. Pteropsids included both true ferns (*Filicinae*) and extinct seed

environments. The Cordaitales, which had tall stems and long, narrow, palmlike leaves, also favoured drier areas. During the Cretaceous and Cenozoic the angiosperms (flowering plants) evolved, producing a diversified flora from which the younger coals developed.

### **Formation processes**

#### **Peat**

Although peat is used as a source of energy, it is not usually considered a coal. It is the precursor material from which coals are derived, and the process by which peat is formed is studied in existing swamps in many parts of the world (e.g., in the Okefenokee Swamp of Georgia, U.S., and along the southwestern coast of New Guinea). The formation of peat is controlled by several factors including (1) the evolutionary development of plant life, (2) the climatic conditions (warm enough to sustain plant growth and wet enough to permit the partial decomposition of the plant material and preserve the peat), and (3) the physical conditions of the area (its geographic position relative to the sea or other bodies of water, rates of subsidence or uplift, and so forth). Warm moist climates are thought to produce broad bands of bright coal. Cooler temperate climates, on the other hand, are thought to produce detrital coal with relatively little bright coal.

Initially, the area on which a future coal seam may be developed must be uplifted so that plant growth can be established. Areas near seacoasts or low-lying areas near streams stay moist enough for peat to form, but elevated swamps (some bogs and moors) can produce peat only if the annual precipitation exceeds annual evaporation and little percolation or drainage occurs. Thick peat deposits necessary for coal formation develop at sites where the following conditions exist: slow, continuous subsidence; the presence of such natural structures as levees, beaches, and bars that give protection from frequent inundation; and a restricted supply of incoming sediments that would interrupt peat formation. In such areas the water may become quite stagnant (except for a few rivers traversing the swamp), and plant material can continue to accumulate. Microorganisms attack the plant material and convert it to peat. Very close to the surface where oxygen is still readily available (aerobic, or oxidizing, conditions), the decomposition of the plant material produces mostly gaseous and liquid products. With increasing depth, however, the conditions become increasingly anaerobic (reducing), and molds and peats develop. The process of peat formation—biochemical coalification—is most active in the upper few metres of a peat deposit. Fungi are not found below about 0.5 metre (about 18 inches) and most forms

(about 30 feet). If either the rate of subsidence or the rate of influx of new sediment increases, the peat will be buried and soon thereafter the coalification process—geochemical coalification—begins. The cycle may be repeated many times, which accounts for the numerous coal seams found in some sedimentary basins.

### Coalification

The general sequence of coalification is from lignite to subbituminous to bituminous to anthracite (*see below* Coal types and ranks). Since microbial activity ceases within a few metres of the Earth's surface, the coalification process must be controlled primarily by changes in physical conditions that take place with depth. Some coal characteristics are determined by events that occur during peat formation—e.g., charcoal-like material in coal is attributed to fires that occurred during dry periods while peat was still forming.

Three major physical factors—duration, increasing temperature, and increasing pressure—may influence the coalification process. In laboratory experiments artificially prepared coals are influenced by the duration of the experiment, but in nature the length of time is substantially longer and the overall effect of time remains undetermined. Low-rank coal (i.e., brown coal) in the Moscow Basin was deposited during Carboniferous time but was not buried deeply and never reached a higher rank. The most widely accepted explanation is that coalification takes place in response to increasing temperature. In general, temperature increases with depth. This geothermal gradient averages about 30 °C (about 85 °F) per kilometre, but the gradient ranges from less than 10 °C (50 °F) per kilometre in regions undergoing very rapid subsidence to more than 100 °C (212 °F) per kilometre in areas of igneous activity. Measurements of thicknesses of sedimentary cover and corresponding coal ranks suggest that temperatures lower than 200 °C (about 390 °F) are sufficient to produce coal of anthracite rank. The effect of increasing pressure due to depth of burial is not considered to cause coalification. In fact, increasing overburden pressure might have the opposite effect if volatile compounds such as methane that must escape during coalification are retained. Pressure may influence the porosity and moisture content of coal.

### Coal types and ranks

Coals may be classified in several ways. One mode of classification is by coal type; such types have some genetic implications because they are based on the organic materials present and the coalification processes that produced the coal. The most useful and widely applied coal-classification schemes are those based on the

degrees of coalification are generally called coal ranks (or classes). In addition to the scientific value of classification schemes of this kind, the determination of rank has a number of practical applications. Many coal properties are in part determined by rank, including the amount of heat produced during combustion, the amount of gaseous products released upon heating, and the suitability of the coals for liquefaction or for producing coke.

## Coal types

### Macerals

Coals contain both organic and inorganic phases. The latter consist either of minerals such as quartz and clays that may have been brought in by flowing water (or wind activity) or of minerals such as pyrite and marcasite that formed in place (authigenic). Some formed in living plant tissues, and others formed later during peat formation or coalification. Some pyrite (and marcasite) is present in micrometre-sized spheroids called framboids (named for their raspberry-like shape) that formed quite early. Framboids are very difficult to remove by conventional coal-cleaning processes.

By analogy to the term *mineral*, British botanist Marie C. Stopes proposed in 1935 the term *maceral* to describe organic constituents present in coals. The word is derived from the Latin *macerare*, meaning "to macerate." (Mineral names often end in "-ite." The corresponding ending for macerals is "-inite.") Maceral nomenclature has been applied differently by some European coal petrologists who studied polished blocks of coal using reflected-light microscopy (their terminology is based on morphology, botanical affinity, and mode of occurrence) and by some North American petrologists who studied very thin slices (thin sections) of coal using transmitted-light microscopy. Various nomenclature systems have been used.



Vitrinite coal maceral  
Courtesy of M. Th.  
Mackowsky,  
Bergbau Forschung,  
Essen, Germany

Three major maceral groups are generally recognized: vitrinite, liptinite (formerly called exinite), and inertinite. The vitrinite group is the most abundant, constituting as much as 50 to 90 percent of many North American coals. Vitrinites are derived primarily from cell walls and woody tissues. They show a wide range of reflectance values (discussed below), but in individual samples these values tend to be intermediate compared with those of the other maceral groups. Several varieties are recognized—e.g., telinite (the brighter parts of vitrinite that make up cell walls) and collinite (clear vitrinite that occupies the spaces between cell walls).



Liptinite coal macerals  
Courtesy of M.Th.  
Mackowsky,  
Bergbauforschung,  
Essen, Germany

The liptinite group makes up 5 to 15 percent of many coals. Liptinites are derived from waxy or resinous plant parts, such as cuticles, spores, and wound resins. Their reflectance values are usually the lowest in an individual sample. Several varieties are recognized, including sporinite (spores are typically preserved as flattened spheroids), cutinite (part of cross sections of leaves, often with crenulated surfaces), and resinite (ovoid and sometimes translucent masses of resin). The liptinites may fluoresce under ultraviolet light, but with increasing rank their optical properties approach those of the vitrinites, and they become indistinguishable.



Inertinite coal macerals  
Courtesy of M.Th.  
Mackowsky,  
Bergbauforschung,  
Essen, Germany

The inertinite group makes up 5 to 40 percent of most coals. Their reflectance values are usually the highest in a given sample. The most common inertinite maceral is fusinite, which has a charcoal-like appearance with obvious cell texture. The cells may be either empty or filled with mineral matter, and the cell walls may have been crushed during compaction (bogen texture). Inertinites are derived from strongly altered or degraded plant material that is thought to have been produced during the formation of peat; in particular, charcoal produced by a fire in a peat swamp is preserved as fusinite.

### Coal rock types

Coals may be classified on the basis of their macroscopic appearance (generally referred to as coal rock type, lithotype, or kohlentype). Four main types are recognized:

1. Vitrain (*Glanzkohle* or *charbon brillant*), which is characterized by a brilliant black lustre and composed primarily of the maceral group vitrinite, which is derived from the woody tissue of large plants. Vitrain is brittle and tends to break into angular fragments; however, thick vitrain layers show conchoidal fractures when broken. Vitrain occurs in narrow, sometimes markedly uniform, bright bands that are about 3 to 10 mm (about 0.1 to 0.4 inch) thick. Vitrain was probably formed under somewhat drier surface conditions than the lithotypes clarain and durain. On burial, stagnant groundwater prevented the complete decomposition of the woody plant tissues.
2. Clarain (*Glanzstreifenkohle* or *charbon semi-brillant*), which has an appearance between those of vitrain and durain and is characterized by alternating bright and dull black laminae.



composed chiefly of the maceral vitrinite and the duller layers of the other maceral groups, liptinite and inertinite. Clarain exhibits a silky lustre less brilliant than that of vitrain. It seems to have originated under conditions that alternated between those in which durain and vitrain were formed.

3. Durain (*Mattkohle* or *charbon mat*), which is characterized by a hard, granular texture and composed of the maceral groups liptinite and inertinite as well as relatively large amounts of inorganic minerals. Durain occurs in layers more than 3 to 10 mm (about 0.1 to 0.4 inch) thick, although layers more than 10 cm (about 4 inches) thick have been recognized. Durains are usually dull black to dark gray in colour. Durain is thought to have formed in peat deposits below water level, where only liptinite and inertinite components resisted decomposition and where inorganic minerals accumulated from sedimentation.
4. Fusain (*Faserkohle* or *charbon fibreux*), which is commonly found in silky and fibrous lenses that are very thin, only millimetres thick and centimetres long. Most fusain is extremely soft and crumbles readily into a fine, sootlike powder that soils the hands. Fusain is composed mainly of fusinite (carbonized woody plant tissue) and semifusinite from the maceral group inertinite, which is rich in carbon and highly reflective. It closely resembles charcoal, both chemically and physically, and is believed to have been formed in peat deposits swept by forest fires, by fungal activity that generated intense heat, or by subsurface oxidation of coal.

### Banded and nonbanded coals



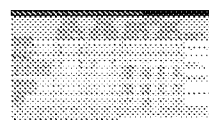
The term *coal type* is also employed to distinguish between banded coals and nonbanded coals (see table). Banded coals contain varying amounts of vitrinite and opaque material. They include bright coal, which contains more than 80 percent vitrinite, and splint coal, which contains more than 30 percent opaque matter. The nonbanded varieties include boghead coal, which has a high percentage of algal remains, and cannel coal with a high percentage of spores. The usage of all the above terms is quite subjective.

### Ranking by coalification

### Hydrocarbon content

chemical composition. Developed in 1837 by the French chemist Henri-Victor Regnault, it was improved in later systems that classified coals on the basis of their hydrogen and carbon content. However, because the relationships between chemistry and other coal properties are complex, such classifications are rarely used for practical purposes today.

### Chemical content and properties



Comparison of coal-rank terminologies by country.  
*Encyclopædia Britannica, Inc.*

The most commonly employed systems of classification are those based on analyses that can be performed relatively easily in the laboratory—for example, determining the percentage of volatile matter lost upon heating to about 950 °C (about 1,750 °F) or the amount of heat released during combustion of the coal under standard conditions. The American Society for Testing and Materials (ASTM) assigns ranks to coals on the basis of fixed carbon content, volatile matter content, and calorific value (see the table). In addition to the major ranks (lignite, subbituminous, bituminous, and anthracite), each rank may be subdivided into coal groups such as high-volatile A bituminous coal. Other designations, such as coking coal and steam coal, have been applied to coals, but they tend to differ from country to country (see illustration).

Coal analyses may be presented in the form of “proximate” and “ultimate” analyses, whose analytical conditions are prescribed by organizations such as the ASTM. A typical proximate analysis includes the moisture, ash, volatile matter, and fixed carbon contents. (Fixed carbon is the material, other than ash, that does not vaporize when heated in the absence of air. It is usually determined by subtracting the sum of the first three values—moisture, ash, and volatile matter—in weight percent from 100 percent.) It is important for economic reasons to know the moisture and ash contents of a coal because they do not contribute to the heating value of a coal. In most cases ash becomes an undesirable residue and a source of pollution, but for some purposes (e.g., use as a chemical feedstock or for liquefaction) the presence of mineral matter may be desirable. Most of the heat value of a coal comes from its volatile matter, excluding moisture, and fixed carbon content. For most coals it is necessary to measure the actual amount of heat released upon combustion (expressed in megajoules per kilogram or British thermal units per pound).

Ultimate analyses are used to determine the carbon, hydrogen, sulfur, nitrogen, ash, oxygen, and moisture contents of a coal. For

These may involve, for example, identifying the forms of sulfur present. Sulfur may occur in the form of sulfide minerals (pyrite and marcasite), sulfate minerals (gypsum), or organically bound sulfur. In other cases the analyses may involve determining the trace elements present (e.g., mercury, chlorine), which may influence the suitability of a coal for a particular purpose or help to establish methods for reducing environmental pollution and so forth.

Virtually all classification systems use the percentage of volatile matter present to distinguish coal ranks. In the ASTM classification, high-volatile A bituminous (and higher ranks) are classified on the basis of their volatile matter content. Coals of lower rank are classified primarily on the basis of their heat values, because of their wide ranges in volatile matter content (including moisture). The agglomerating character of a coal refers to its ability to soften and swell when heated and to form cokelike masses that are used in the manufacture of steel. The most suitable coals for agglomerating purposes are in the bituminous rank.

### **Structure and properties of coal**

#### **Organic compounds**

The plant material from which coal is derived is composed of a complex mixture of organic compounds, including cellulose, lignin, fats, waxes, and tannins. As peat formation and coalification proceed, these compounds, which have more or less open structures, are broken down, and new compounds—primarily aromatic (benzenelike) and hydroaromatic—are produced. In vitrinite these compounds are connected by cross-linking oxygen, sulfur, and molecules such as methylene. During coalification, volatile phases rich in hydrogen and oxygen (e.g., water, carbon dioxide, and methane) are produced and escape from the mass; hence, the coal becomes progressively richer in carbon. The classification of coal by rank is based on these changes—i.e., as coalification proceeds, the amount of volatile matter gradually decreases and the amount of fixed carbon increases. As volatiles are expelled, more carbon-to-carbon linkages occur in the remaining coal until, having reached the anthracite rank, it takes on many of the characteristics of the end product of the metamorphism of carbonaceous material—namely, graphite. Coals pass through several structural states as the bonds between the aromatic nuclei increase.

#### **Properties**

Many of the properties of coal are strongly rank-dependent.

presence of mineral matter also influence its properties. Several techniques have been developed for studying the physical and chemical properties of coal, including density measurements, X-ray diffraction, scanning and transmission electron microscopy, infrared spectrophotometry, mass spectroscopy, gas chromatography, thermal analysis, and electrical, optical, and magnetic measurements.

### **Density**

Knowledge of the physical properties of coal is important in coal preparation and utilization. For example, coal density ranges from approximately 1.1 to about 1.5 megagrams per cubic metre, or grams per cubic centimetre (1 megagram per cubic metre equals 1 gram per cubic centimetre). Coal is slightly denser than water (1.0 megagram per cubic metre) and significantly less dense than most rock and mineral matter (e.g., shale has a density of about 2.7 megagrams per cubic metre and pyrite of 5.0 megagrams per cubic metre). Density differences make it possible to improve the quality of a coal by removing most of the rock matter and sulfide-rich fragments by means of heavy liquid separation (fragments with densities greater than about 1.5 megagrams per cubic metre settle out while the coal floats on top of the liquid). Devices such as cyclones and shaker tables also separate coal particles from rock and pyrite on the basis of their different densities.

### **Porosity**

Coal density is controlled in part by the presence of pores that persist throughout coalification. Measurement of pore sizes and pore distribution is difficult; however, there appear to be three size ranges of pores: (1) macropores (diameter greater than 50 nanometres), (2) mesopores (diameter 2 to 50 nanometres), and (3) micropores (diameter less than 2 nanometres). (One nanometre is equal to  $10^{-9}$  metre.) Most of the effective surface area of a coal—about 200 square metres per gram—is not on the outer surface of a piece of coal but is located inside the coal in its pores. The presence of pore space is important in the production of coke, gasification, liquefaction, and the generation of high-surface-area carbon for purifying water and gases. From the standpoint of safety, coal pores may contain significant amounts of adsorbed methane that may be released during mining operations and form explosive mixtures with air. The risk of explosion can be reduced by adequate ventilation during mining or by prior removal of coal-bed methane.

### **Reflectivity**

i.e., its ability to reflect light. Reflectivity is measured by shining a beam of monochromatic light (with a wavelength of 546 nanometres) on a polished surface of the vitrinite macerals in a coal sample and measuring the percentage of the light reflected with a photometer. Vitrinite is used because its reflectivity changes gradually with increasing rank. Fusinite reflectivities are too high due to its origin as charcoal, and liptinites tend to disappear with increasing rank. Although little of the incident light is reflected (ranging from a few tenths of a percent to 12 percent), the value increases with rank and can be used to determine the rank of most coals without measuring the percentage of volatile matter present.

The study of coals (and coaly particles called phyterals) in sedimentary basins containing oil and/or gas reveals a close relationship between coalification and the maturation of liquid and gaseous hydrocarbons. During the initial stages of coalification (to a reflectivity of almost 0.5 and near the boundary between subbituminous and high-volatile C bituminous coal), hydrocarbon generation produces chiefly methane. The maximum generation of liquid petroleum occurs during the development of high-volatile bituminous coals (in the reflectivity range from roughly 0.5 to about 1.3). With increasing depth and temperature, petroleum liquids break down and, finally, only natural gas (methane) remains. Geologists can use coal reflectivity to anticipate the potential for finding liquid or gaseous hydrocarbons as they explore for petroleum.

### **Other properties**

Other properties, such as hardness, grindability, ash-fusion temperature, and free-swelling index (a visual measurement of the amount of swelling that occurs when a coal sample is heated in a covered crucible), may affect coal mining and preparation, as well as the way in which a coal is utilized. Hardness and grindability determine the kinds of equipment used for mining, crushing, and grinding coals in addition to the amount of power consumed in their operation. Ash-fusion temperature influences furnace design and operating conditions. The free-swelling index provides preliminary information concerning the suitability of a coal for coke production.

### **World distribution of coal**

#### **General occurrence**



Coal is a widespread resource of energy and chemicals. Although terrestrial plants necessary for the development of coal did not become

occurrences on Earth.  
Encyclopedia  
Britannica, Inc.

sedimentary basins containing Carboniferous and younger rocks are known on virtually every continent, including Antarctica. The presence of large coal deposits in regions that now have arctic or subarctic climates (such as Alaska and Siberia) is due to climatic changes and to the tectonic motion of crustal plates that moved ancient continental masses over the Earth's surface, sometimes through subtropical and even tropical regions. The absence of coal in some areas (such as Greenland and much of northern Canada) results from the fact that the rocks found there are older than Carboniferous period and that these regions, known as shields, lacked the abundant terrestrial plant life needed for the formation of major coal deposits.

### Resources and reserves

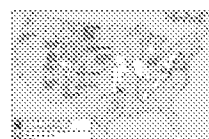
World coal reserves and resources are difficult to assess. Although some of the difficulty stems from the lack of accurate data for individual countries, two fundamental problems make these estimates difficult and subjective. The first problem concerns differences in the definition of terms such as *proven reserves* (generally only those quantities that are recoverable) and *geological resources* (generally the total amount of coal present, whether or not recoverable at present).

The proven reserves for any commodity should provide a reasonably accurate estimate of the amount that can be recovered under existing operating and economic conditions. To be economically mineable, a coal bed must have a minimum thickness (about 0.6 metre; 2 feet) and be buried less than some maximum depth (roughly 2,000 metres; 6,600 feet) below the Earth's surface. These values of thickness and depth are not fixed but change with coal quality, demand, the ease with which overlying rocks can be removed (in surface mining) or a shaft sunk to reach the coal seam (in underground mining), and so forth. The development of new mining techniques may increase the amount of coal that can be extracted relative to the amount that cannot be removed. For example, in underground mining (which accounts for about 60 percent of world coal production), conventional mining methods leave behind large pillars of coal to support the overlying rocks and recover only about half of the coal present. On the other hand, longwall mining, in which the equipment removes continuous parallel bands of coal, may recover nearly all the coal present.



The second problem, which concerns the estimation of reserves, is the rate at which a commodity is consumed. When considering the

amount of coal resources. At present rates of consumption, world coal reserves should last more than 300-500 years. The table indicates the present quantity of proven coal reserves. A large amount of additional coal is present in the Earth but cannot be recovered at this time. These resources, sometimes called "geologic resources," are even more difficult to estimate, but they are thought to be as much as 15 times greater than the amount of proven reserves.



Coal-bearing areas of the conterminous

United States.

Encyclopedia

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One ton of coal equivalent equals 1 metric ton (2,205 pounds) of coal with a heating value of 29.3 megajoules per kilogram (12,600 British thermal units per pound). These values suggest that the United States has the largest amount of recoverable coal. Nearly 80 percent of the world's recoverable coal resources are controlled by seven countries: the United States (about 27 percent), Russia (about 17 percent), China (about 13 percent), India (about 10 percent), South Africa (about 5 percent), Ukraine (about 4 percent), and Kazakhstan (about 3 percent).

Otto C. Kopp

### Additional Reading

G.H. TAYLOR et al., *Organic Petrology* (1998); BP/Amoco, *BP Statistical Review of World Energy* (2006); and the World Energy Council, *Survey of Energy Resources* (2004), provide data on proven reserves.

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## petroleum

Encyclopædia Britannica Article

complex mixture of hydrocarbons that occur in the Earth in liquid, gaseous, or solid forms. The term is often restricted to the liquid form, commonly called crude oil, but as a technical term it also includes natural gas and the viscous or solid form known as bitumen. The liquid and gaseous phases of petroleum constitute the most important of the primary fossil fuels.

Liquid and gaseous hydrocarbons are so intimately associated in nature that it has become customary to shorten the expression "petroleum and natural gas" to "petroleum" when referring to both. The word petroleum (literally "rock oil" from the Latin *petra*, "rock" or "stone," and *oleum*, "oil") was first used in 1556 in a treatise published by the German mineralogist Georg Bauer, known as Georgius Agricola.

### History of use

#### Exploitation of surface seeps

Small surface occurrences of petroleum in the form of natural gas and oil seeps have been known from early times. The ancient Sumerians, Assyrians, and Babylonians used crude oil and asphalt ("pitch") collected from large seeps at Tuttul (modern-day Hit) on the Euphrates for many purposes more than 5,000 years ago. Liquid oil was first used as a medicine by the ancient Egyptians, presumably as a wound dressing, liniment, and laxative.

Oil products were valued as weapons of war in the ancient world. The Persians used incendiary arrows wrapped in oil-soaked fibres at the siege of Athens in 480 BC. Early in the Christian era the Arabs and Persians distilled crude oil to obtain flammable products for military purposes. Probably as a result of the Arab invasion of Spain, the industrial art of distillation into illuminants became available in western Europe by the 12th century.

Several centuries later, Spanish explorers discovered oil seeps in present-day Cuba, Mexico, Bolivia, and Peru. In North America oil seeps were plentiful and were noted by early explorers in what are now New York and Pennsylvania, where the Indians were reported to have used the oil for medicinal purposes.

#### Extraction from underground reservoirs

States and in many other countries was little improved over that known by the early Greeks and Romans. The need for better illumination that accompanied the increasing development of urban centres made it necessary to search for new sources of oil, especially since whales, which had long provided fuel for lamps, were becoming harder and harder to find. By the mid-19th century kerosene, or coal oil, derived from coal was in common use in both North America and Europe.

The Industrial Revolution brought on an ever-growing demand for a cheaper and more convenient source of lubricants as well as illuminating oil. It also required better sources of energy. Energy had previously been provided by human and animal muscle and later by the combustion of such solid fuels as wood, peat, and coal. These were collected with considerable effort and laboriously transported to the site where the energy source was needed. Liquid petroleum, on the other hand, was a more easily transportable source of energy. Oil was a much more concentrated and flexible form of fuel than anything previously available.

The stage was set for the first well specifically drilled for oil, a project undertaken by Edwin L. Drake in northwestern Pennsylvania. The completion of the well in August 1859 established the groundwork for the petroleum industry and ushered in the closely associated modern industrial age. Within a short time inexpensive oil from underground reservoirs was being processed at already existing coal-oil refineries, and by the end of the century oil fields had been discovered in 14 states from New York to California and from Wyoming to Texas. During the same period, oil fields were found in Europe and East Asia as well.

### **Significance of oil in modern times**

At the beginning of the 20th century the Industrial Revolution had progressed to the extent that the use of refined oil for illuminants ceased to be of primary importance. The oil industry became the major supplier of energy largely because of the advent of the automobile. Although oil constitutes a major petrochemical feedstock, its primary importance is as an energy source on which the world economy depends.

The significance of oil as a world energy source is difficult to overdramatize. The growth in energy production during the 20th century is unprecedented, and increasing oil production has been by far the major contributor to that growth. Every day an immense

from producers to consumers. The production and consumption of oil is of vital importance to international relations and has frequently been a decisive factor in the determination of foreign policy. The position of a country in this system depends on its production capacity as related to its consumption. The possession of oil deposits is sometimes the determining factor between a rich and a poor country. For any country, however, the presence or absence of oil has a major economic consequence.

On a time scale within the span of prospective human history, the utilization of oil as a major source of energy will be a transitory affair of about 100 years. Nonetheless, it will have been an affair of profound importance to world industrialization.

### **Properties of oil**

#### **Chemical composition**

##### **Hydrocarbon content**

Although oil consists basically of compounds of only two elements, carbon and hydrogen, these elements form a large variety of complex molecular structures. Regardless of physical or chemical variations, however, almost all crude oil ranges from 82 to 87 percent carbon by weight and 12 to 15 percent hydrogen. The more viscous bitumens generally vary from 80 to 85 percent carbon and from 8 to 11 percent hydrogen.

Crude oil can be grouped into three basic chemical series: paraffins, naphthenes, and aromatics. Most crude oils are mixtures of these three series in various and seemingly endless proportions. No two crude oils from different sources are completely identical.

The paraffin series of hydrocarbons, also called the methane ( $\text{CH}_4$ ) series, comprises the most common hydrocarbons in crude oil. It is a saturated straight-chain series that has the general formula  $\text{C}_n\text{H}_{2n+2}$ , in which C is carbon, H is hydrogen, and  $n$  is an integer. The paraffins that are liquid at normal temperatures but boil between  $40^\circ$  and  $200^\circ$  C (approximately between  $100^\circ$  and  $400^\circ$  F) are the major constituents of gasoline. The residues obtained by refining lower-density paraffins are both plastic and solid paraffin waxes.

The naphthene series has the general formula  $\text{C}_n\text{H}_{2n}$  and is a saturated closed-ring series. This series is an important part of all

residues from the higher boiling-point ranges. For this reason, the series is generally heavier. The residue of the refinery process is an asphalt, and the crude oils in which this series predominates are called asphalt-base crudes.

The aromatic series has the general formula  $C_nH_{2n-6}$  and is an unsaturated closed-ring series. Its most common member, benzene ( $C_6H_6$ ), is present in all crude oils, but the aromatics as a series generally constitute only a small percentage of most crudes.

### **Nonhydrocarbon content**

In addition to the practically infinite mixtures of hydrocarbon compounds that form crude oil, sulfur, nitrogen, and oxygen are usually present in small but often important quantities. Sulfur is the third most abundant atomic constituent of crude oils. It is present in the medium and heavy fractions of crude oils. In the low and medium molecular ranges, sulfur is associated only with carbon and hydrogen, while in the heavier fractions it is frequently incorporated in the large polycyclic molecules that also contain nitrogen and oxygen. The total sulfur in crude oil varies from below 0.05 percent (by weight), as in some Pennsylvania oils, to about 2 percent for average Middle Eastern crudes and up to 5 percent or more in heavy Mexican or Mississippi oils. Generally, the higher the specific gravity of the crude oil, the greater is its sulfur content. The excess sulfur is removed from crude oil during refining, because sulfur oxides released into the atmosphere during the combustion of oil would constitute a major pollutant.

The oxygen content of crude oil is usually less than 2 percent by weight and is present as part of the heavier hydrocarbon compounds in most cases. For this reason, the heavier oils contain the most oxygen. Nitrogen is present in almost all crude oils, usually in quantities of less than 0.1 percent by weight. Sodium chloride also occurs in most crudes and is usually removed like sulfur.

Many metallic elements are found in crude oils, including most of those that occur in seawater. This is probably due to the close association between seawater and the organic forms from which oil is generated. Among the most common metallic elements in oil are vanadium and nickel, which apparently occur in organic combinations as they do in living plants and animals.

*Crude oil also may contain a small amount of decay-resistant*

spores, resins, coal, and various other remnants of former life.

### **Physical properties**

Oil consists of a closely related series of complex hydrocarbon compounds that range from gasoline to heavy solids. The various mixtures that constitute crude oil can be separated by distillation under increasing temperatures into such components as (from light to heavy) gasoline, kerosene, gas oil, lubricating oil, residual fuel oil, bitumen, and paraffin.

Crude oils vary greatly in their chemical composition. Because they consist of mixtures of thousands of hydrocarbon compounds, their physical properties such as colour, specific gravity, and viscosity also vary widely.

### **Specific gravity**

Crude oil is immiscible with and lighter than water; hence it floats. Crude oils are generally classified as tar sands, heavy oils, and medium and light oils on the basis of specific gravity (*i.e.*, the ratio of the weight of equal volumes of the oil and pure water at standard conditions, with pure water considered to equal 1) and relative mobility. Tar sands contain immobile oil, which does not flow into a well bore (see below). Heavy crude oils have enough mobility that, given time, they can be obtained through a well bore in response to enhanced recovery methods. The more mobile medium and light oils are recoverable through production wells.

The widely used American Petroleum Institute (API) gravity scale is based on pure water, with an arbitrarily assigned API gravity of 10°. Liquids lighter than water, such as oil, have API gravities numerically greater than 10. Crude oils below 20° API gravity are usually considered heavy, whereas the conventional crudes with API gravities between 20° and 25° are regarded as medium, with light oils ranging above 25°.

### **Boiling and freezing points**

Because oil is always at a temperature above the boiling point of some of its compounds, the more volatile constituents constantly escape into the atmosphere unless confined. It is impossible to refer to a common boiling point for crude oil because of the widely differing boiling points of its numerous compounds, some of which may boil at temperatures too high to be measured.

point for a crude oil because the individual compounds solidify at different temperatures. However, the pour point—the temperature below which crude oil becomes plastic and will not flow—is important to recovery and transport and is always determined. Pour points range from 32° C to below -57° C.

### **Measurement systems**

In the United States, crude oil is measured in barrels of 42 gallons each; the weight per barrel of API 30° light oil would be about 306 pounds. In many other countries, crude oil is measured in metric tons. For oil having the same gravity, a metric ton is equal to approximately 252 imperial gallons or about 7.2 U.S. barrels.

### **Origin of crude oil**

#### **Formation process**

##### **From planktonic remains to kerogen**

Although it is recognized that the original source of carbon and hydrogen was in the materials that made up the primordial Earth, it is generally accepted that these two elements have had to pass through an organic phase to be combined into the varied complex molecules recognized as crude oil. The organic material that is the source of most oil has probably been derived from single-celled planktonic (free-floating) plants, such as diatoms and blue-green algae, and single-celled planktonic animals, such as foraminifera, which live in aquatic environments of marine, brackish, or fresh water. Such simple organisms are known to have been abundant long before the Paleozoic Era, which began some 540,000,000 years ago.

Rapid burial of the remains of the single-celled planktonic plants and animals within fine-grained sediments effectively preserved them. This provided the organic materials, the so-called protopetroleum, for later diagenesis (*i.e.*, the series of processes involving biological, chemical, and physical changes) into true petroleum.

The first, or immature, stage of petroleum formation is dominated by biological activity and chemical rearrangement, which convert organic matter to kerogen. This dark-coloured, insoluble product of bacterially altered plant and animal detritus is the source of most hydrocarbons generated in the later stages. During the first stage, biogenic methane is the only hydrocarbon generated in commercial

process of decomposition of organic matter carried out by anaerobic microorganisms (those capable of living in the absence of free oxygen).

### **From kerogen to petroleum**

Deeper burial by continuing sedimentation, increasing temperatures, and advancing geologic age result in the mature stage of petroleum formation, during which the full range of petroleum compounds is produced from kerogen and other precursors by thermal degradation and cracking (the process by which heavy hydrocarbon molecules are broken up into lighter molecules). Depending on the amount and type of organic matter, oil generation occurs during the mature stage at depths of about 760 to 4,880 metres (2,500 to 16,000 feet) at temperatures between 65° and 150° C. This special environment is called the “oil window.” In areas of higher than normal geothermal gradient (increase in temperature with depth), the oil window exists at shallower depths in younger sediments but is narrower. Maximum oil generation occurs from depths of 2,000 to 2,900 metres. Below 2,900 metres primarily wet gas, a type of gas containing liquid hydrocarbons known as natural gas liquids, is formed.

Approximately 90 percent of the organic material in sedimentary source rocks is dispersed kerogen. Its composition varies, consisting as it does of a range of residual materials whose basic molecular structure takes the form of stacked sheets of aromatic hydrocarbon rings in which atoms of sulfur, oxygen, and nitrogen also occur. Attached to the ends of the rings are various hydrocarbon compounds, including normal paraffin chains. The mild heating of the kerogen in the oil window of a source rock over long periods of time results in the cracking of the kerogen molecules and the release of the attached paraffin chains. Further heating, perhaps assisted by the catalytic effect of clay minerals in the source rock matrix, may then produce soluble bitumen compounds, followed by the various saturated and unsaturated hydrocarbons, asphaltenes, and others of the thousands of hydrocarbon compounds that make up crude oil mixtures.

At the end of the mature stage, below about 4,880 metres, depending on the geothermal gradient, kerogen becomes condensed in structure and chemically stable. In this environment, crude oil is no longer stable and the main hydrocarbon product is dry thermal methane gas.

### Origin in source beds

Knowing the maximum temperature reached by a potential source rock during its geologic history helps in estimating the maturity of the organic material contained within it. Also, this information may indicate whether a region is gas-prone, oil-prone, both, or neither. The techniques employed to assess the maturity of potential source rocks in core samples include measuring the degree of darkening of fossil pollen grains and the colour changes in conodont fossils. In addition, geochemical evaluations can be made of mineralogical changes that were also induced by fluctuating paleotemperatures. In general, there appears to be a progressive evolution of crude oil characteristics from geologically younger, heavier, darker, more aromatic crudes to older, lighter, paler, more paraffinic types. There are, however, many exceptions to this rule, especially in regions with high geothermal gradients.

Accumulations of petroleum are usually found in relatively coarse-grained, permeable, and porous sedimentary reservoir rocks that contain little, if any, insoluble organic matter. It is unlikely that the vast quantities of oil now present in some reservoir rocks could have been generated from material of which no trace remains. Therefore, the site where commercial amounts of oil originated apparently is not always identical to the location at which they are ultimately discovered.

Oil is believed to have been generated in significant volumes only in fine-grained sedimentary rocks (usually clays, shales, or clastic carbonates) by geothermal action on kerogen, leaving an insoluble organic residue in the source rock. The release of oil from the solid particles of kerogen and its movement in the narrow pores and capillaries of the source rock is termed primary migration.

Accumulating sediments can provide energy to the migration system. Primary migration may be initiated during compaction as a result of the pressure of overlying sediments. Continued burial causes clay to become dehydrated by the removal of water molecules that were loosely combined with the clay minerals. With increasing temperature, the newly generated hydrocarbons may become sufficiently mobile to leave the source beds in solution, suspension, or emulsion with the water being expelled from the compacting molecular lattices of the clay minerals. The hydrocarbon molecules would compose only a very small part of the migrating fluids, a few hundred parts per million.

### Migration through carrier beds





Figure 1: Principal types

of traps.

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The hydrocarbons expelled from a source bed next move through the wider pores of carrier beds (e.g., sandstones or carbonates) that are coarser-grained and more permeable. This movement is termed secondary migration. The distinction

between primary and secondary migration is based on pore size and rock type. In some cases, oil may migrate through such permeable carrier beds until it is trapped by a permeability barrier and forms an oil accumulation (Figure 1). In others, the oil may continue its migration until it becomes a seep on the surface of the Earth, where it will be broken down chemically by oxidation and bacterial action.

Since nearly all pores in subsurface sedimentary formations are water-saturated, the migration of oil takes place in an aqueous environment. Secondary migration may result from active water movement or can occur independently, either by displacement or by diffusion. Because the specific gravity of the water in the sedimentary formation is considerably higher than that of oil, the oil will float to the surface of the water in the course of geologic time and accumulate in the highest portion of a trap.

### Accumulation in reservoir beds

The porosity (volume of pore spaces) and permeability (capacity for transmitting fluids) of carrier and reservoir beds are important factors in the migration and accumulation of oil. Most petroleum accumulations have been found in clastic reservoirs (sandstones and siltstones). Next in number are the carbonate reservoirs (limestones and dolomites). Accumulations of petroleum also occur in shales and igneous and metamorphic rocks because of porosity resulting from fracturing, but such reservoirs are relatively rare. Porosities in reservoir rocks usually range from about 5 to 30 percent, but all available pore space is not occupied by petroleum. A certain amount of residual formation water cannot be displaced and is always present.

Reservoir rocks may be divided into two main types: (1) those in which the porosity and permeability is primary, or inherent, and (2) those in which they are secondary, or induced. Primary porosity and permeability are dependent on the size, shape, and grading and packing of the sediment grains and also on the manner of their initial consolidation. Secondary porosity and permeability result from postdepositional factors, such as solution, recrystallization, fracturing, weathering during temporary exposure at the Earth's

## Oil traps

After secondary migration in carrier beds, oil finally collects in a trap. The fundamental characteristic of a trap is an upward convex form of porous and permeable reservoir rock that is sealed above by a denser, relatively impermeable cap rock (e.g., shale or evaporites). The trap may be of any shape, the critical factor being that it is a closed, inverted container. A rare exception is hydrodynamic trapping, in which high water saturation of low-permeability sediments reduces hydrocarbon permeability to near zero, resulting in a water block and an accumulation of petroleum down the structural dip of a sedimentary bed below the water in the sedimentary formation.

## Structural traps



Figure 1: Principal types of traps.  
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Traps can be formed in many ways (Figure 1). Those formed by tectonic events, such as folding or faulting of rock units, are called structural traps. The most common structural traps are anticlines, upfolds of strata that appear as ovals on the horizontal planes of geologic maps. About 80 percent of the world's petroleum has been found in anticlinal traps. Most anticlines were produced by lateral pressure, but some have resulted from the draping and subsequent compaction of accumulating sediments over topographic highs. The closure of an anticline is the vertical distance between its highest point and the spill plane, the level at which the petroleum can escape if the trap is filled beyond capacity. Some traps are filled with petroleum to their spill plane, but others contain considerably smaller amounts than they can accommodate on the basis of their size.

Another kind of structural trap is the fault trap. Here, rock fracture results in a relative displacement of strata that forms a barrier to petroleum migration. A barrier can occur when an impermeable bed is brought into contact with a carrier bed. Sometimes the faults themselves provide a seal against “updip” migration when they contain impervious clay gouge material between their walls. Faults and folds often combine to produce traps, each providing a part of the container for the enclosed petroleum. Faults can, however, allow the escape of petroleum from a former trap if they breach the cap rock seal.

Other structural traps are associated with salt domes. Such traps are formed by the upward movement of salt masses from deeply buried evaporite beds, and they occur along the folded or faulted

or draped sediments.

### **Stratigraphic traps**

A second major class of oil traps is the stratigraphic trap. It is related to sediment deposition or erosion and is bounded on one or more sides by zones of low permeability. Because tectonics ultimately control deposition and erosion, however, few stratigraphic traps are completely without structural influence. The geologic history of most sedimentary basins contains the prerequisites for the formation of stratigraphic traps. Typical examples are fossil carbonate reefs, marine sandstone bars, and deltaic distributary channel sandstones. When buried, each of these geomorphic features provides a potential reservoir, which is often surrounded by finer-grained sediments that may act as source or cap rocks.

Sediments eroded from a landmass and deposited in an adjacent sea change from coarse- to fine-grained with increasing depth of water and distance from shore. Permeable sediments thus grade into impermeable sediments, forming a permeability barrier that eventually could trap migrating petroleum.

There are many other types of stratigraphic traps. Some are associated with the many transgressions and regressions of the sea that have occurred over geologic time and the resulting deposits of differing porosities. Others are caused by processes that increase secondary porosity, such as the dolomitization of limestones or the weathering of strata once located at the Earth's surface.

### **World distribution of oil**

#### **Location of reserves**

#### **Oil fields**

Two overriding principles apply to world petroleum production. First, most petroleum is contained in a few large fields, but most fields are small. Second, as exploration progresses, the average size of the fields discovered decreases, as does the amount of petroleum found per unit of exploratory drilling. In any region, the large fields are usually discovered first.

Since exploration for oil began during the early 1860s, some 50,000 oil fields have been discovered. More than 90 percent of these fields

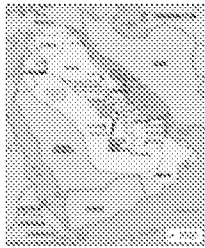


Figure 2: Major oil fields of the Arabian-Iranian basin region.  
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production. The two largest classes of fields are the supergiants, fields with 5,000,000,000 or more barrels of ultimately recoverable oil, and world-class giants, fields with 500,000,000 to 5,000,000,000 barrels of ultimately recoverable oil. Fewer than 40 supergiant oil fields have been found worldwide, yet these fields originally contained about one-half of all the oil so far discovered. The Arabian-Iranian sedimentary basin in the Persian Gulf region contains two-thirds of these supergiant fields (Figure 2). The remaining supergiants are distributed as follows: two in the United States, two in Russia, two in Mexico, one in Libya, one in Algeria, one in Venezuela, and two in China.

The nearly 280 world-class giant fields thus far discovered, plus the supergiants, account for about 80 percent of the world's known recoverable oil. There are, in addition, approximately 1,000 known large oil fields that initially contained between 50,000,000 and 500,000,000 barrels. These fields account for some 14 to 16 percent of the world's known oil. Less than 5 percent of the known fields originally contained roughly 95 percent of the world's known oil.

### **Sedimentary basins**

Giant petroleum fields and significant petroleum-producing sedimentary basins are closely associated. In some basins, huge amounts of petroleum apparently have been generated because perhaps only about 10 percent of the generated petroleum is trapped and preserved. The Arabian-Iranian sedimentary basin is predominant because it contains more than 20 supergiant fields. No other basin has more than one such field. In 20 of the 26 most significant oil-containing basins, the 10 largest fields originally contained more than 50 percent of the known recoverable oil. Known world oil reserves are concentrated in a relatively small number of giant fields in a few sedimentary basins.

Worldwide, approximately 600 sedimentary basins are known to exist. About 160 of these have yielded oil, but only 26 are significant producers and 7 of these account for more than 65 percent of total known oil. Exploration has occurred in another 240 basins, but discoveries of commercial significance have not been made.

Current geologic understanding can usually distinguish between geologically favourable and unfavourable conditions for oil accumulation early in the exploration cycle. Thus, only a relatively few exploratory wells may be necessary to indicate whether a region is likely to contain significant amounts of oil. Modern petroleum exploration is an efficient process. If giant fields exist, it is likely that most of the oil in a region will be found by the first 50 to 250 exploratory wells. This number may be exceeded if there is a much greater than normal amount of major prospects or if exploration drilling patterns are dictated by either political or unusual technological considerations. Thus, while undiscovered commercial oil fields may exist in some of the 240 explored but seemingly barren basins, it is unlikely that they will be of major importance since the largest are normally found early in the exploration process.

The remaining 200 basins have had little or no exploration, but they have had sufficient geologic study to indicate their dimensions, amount and type of sediments, and general structural character. Most of the underexplored (or frontier) basins are located in difficult environments, such as polar regions or submerged continental margins. The larger sedimentary basins—those containing more than 833,000 cubic kilometres (200,000 cubic miles) of sediments—account for some 70 percent of known world petroleum. Future exploration will have to involve the smaller basins as well as the more expensive and difficult frontier basins.

### Status of the world oil supply

The first 200,000,000,000 barrels of world oil were produced in 109 years from 1859 to 1968. Since that time world oil production rates have stabilized at a rate of about 22,000,000,000 barrels a year.



Table 1 shows the broad distribution of the world oil supply. Reserves are identified quantities of “in-place” petroleum that are considered recoverable under current economic and technological conditions. Estimated by petroleum engineers and geologists using drilling and production data along with other subsurface information, these figures are revised to include projected field growth as development progresses. Petroleum reserves are reported by oil companies and by some governments, and such data are compiled by the U.S. Department of Energy and the U.S. Geological Survey, as well as by oil industry trade journals.

*Oil and gas reserves, 1990. Source: U.S. Geological Survey.*

known production and reserve data into untested sediments of similar geology. A most likely consensus estimate was established, as was a range with upper and lower yield limits at 5 and 95 percent probabilities. The range for undiscovered oil resources assessed for the whole world is 275,000,000,000 to 1,469,000,000,000 barrels.



The most likely total world oil endowment is about 2,390,000,000,000 barrels, as seen in Table 1. Of this amount, 77 percent has already been discovered and 30 percent has already been produced and consumed. If this estimate proves to be reasonably accurate, current relatively stabilized world oil-production volumes could be sustained to about the middle of the 21st century, at which time a shortage of conventional oil resources would force a production decline.

The Middle East is thought to have had an estimated 41 percent of the world's total oil endowment. North America is a distant second but has already produced almost half of its total oil. Eastern Europe, because of the large deposits in Russia, is well endowed with oil. Western Europe is not, with most of its oil under the North Sea. Likewise, Africa, Asia, and South America are thought to have only relatively moderate amounts of oil. It is interesting to note that a large undiscovered oil resource is believed to exist in North America, which has many frontier basins. Both the Middle East and eastern Europe, however, are also thought to contain significant oil prospects.

### Major oil-producing countries



There are 18 countries that are believed to have had an original oil endowment exceeding 20,000,000,000 barrels, as shown in Table 2. The table also serves to show the concentration of world oil. These 18 countries have accounted for 86 percent of the world's oil production. They hold 94 percent of its reserves. Significantly, they are projected to have 82 percent of the world's remaining undiscovered oil resources. As can be seen, regions geologically favourable to the generation and deposition of oil are fairly rare. The 18 countries listed are estimated to have contained 89 percent of the world's original oil endowment.

### Saudi Arabia

Saudi Arabia, shown in Figure 2, is thought to have

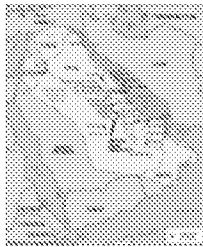


Figure 2: Major oil fields of the Arabian-Iranian basin region.  
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country. The discovery that transformed Saudi Arabia into a leading oil country was the Al-Ghawar field. Discovered in 1948, this field has proved to be the world's largest, containing 82,000,000,000 barrels. Another important discovery was the Saffaniyah offshore field in the Persian Gulf. It is the third largest oil field in the world and the largest offshore. Saudi Arabia has eight other supergiant oil fields. Thus, it has the largest oil reserve in the world, not to mention significant potential for additional discoveries.

## Russia

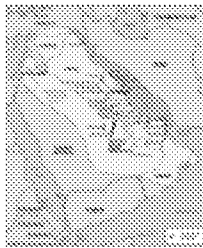


Figure 2: Major oil fields of the Arabian-Iranian basin region.  
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Russia is thought to possess the best potential for new discoveries. Also, it has significant reserves. Russian oil is derived from many sedimentary basins within the vast country, while Saudi Arabian fields, as well as many other Middle Eastern fields, are located in the great Arabian-Iranian basin (Figures 2 and 3). Russia has two supergiant oil fields, Samotlor and Romashkino. Production from these fields is on the decline, bringing total Russian oil output down with them. The best prospects for new Russian discoveries appear to exist in the difficult and expensive frontier areas.



Figure 3: Sedimentary basins and major oil and gas fields of Europe, Russia, Transcaucasia, and ...  
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## United States, Mexico, and Canada

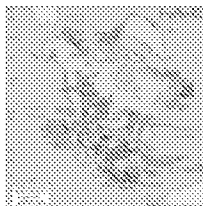


Figure 4: Sedimentary basins and major oil and gas fields of North America.

North America also has many sedimentary basins; they are shown in Figure 4. Basins in the United States have been intensively explored and their oil resources developed. More than 33,000 oil fields have been found, but only two are supergiants (Prudhoe Bay in the North Slope region of Alaska and East Texas). Cumulatively, the United States has produced more oil than any other country but

accounted for approximately 17 percent of U.S. oil production during the mid-1980s, is in decline. This situation, coupled with declining oil production in the conterminous United States, has contributed to a significant drop in domestic oil output. Mexico has produced only about one-fifth of its estimated total oil endowment. With two supergiant fields (Cantarell offshore of Campeche state and Bermudez in Tabasco state) and with substantial remaining reserves and resources, it will be able to sustain current production levels well into the 21st century. Conversely, Canada, with considerably smaller oil reserves and most of its undiscovered resource potential in remote regions, is unlikely to be able to sustain current production levels beyond the 1990s. Canada's largest oil field is Hibernia, discovered off Newfoundland in 1979. This giant field has yet to be developed.

### **Iraq, Kuwait, and Iran**

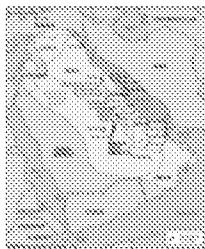


Figure 2: Major oil fields of the Arabian-Iranian basin region.  
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The Middle Eastern countries of Iraq, Kuwait, and Iran are each estimated to have had an original oil endowment in excess of 100,000,000,000 barrels. These countries have a number of supergiant fields, all of which are located in the Arabian-Iranian basin, including Kuwait's Al-Burqan field (Figure 2). Al-Burqan is the world's second largest oil field, having originally contained 75,000,000,000 barrels of recoverable oil. Iraq possesses a significant potential for additional oil discoveries.

### **United Kingdom**

The United Kingdom is an important North Sea exporter; however, as its undiscovered resource potential appears somewhat limited, it may require more of its oil output for internal use in the future.

### **Undiscovered resources**

With an estimated 77 percent of the world's total recoverable oil endowment having already been discovered, the remaining 23 percent, mostly located in smaller fields or in more difficult environments, is expected to become ever more expensive to find and to recover. More than 11,000 man-years were required to construct the largest of the North Sea gravity platforms, making capital costs per daily oil production as much as 40 times the costs in the Middle East. A guayed tower constructed in more than 300 metres of water in the Gulf of Mexico has been estimated to



East. As oil exploitation moves into deeper waters or under Arctic ice, the cost will further escalate and will be reflected in the world economy.

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## Microbial production of poly-D-3-hydroxybutyrate from CO<sub>2</sub>

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**Abstract** This short review covers the biotechnological aspects of the production of poly-D-3-hydroxybutyric acid, P(3HB), from H<sub>2</sub>, O<sub>2</sub> and CO<sub>2</sub> by autotrophic culture of the hydrogen-oxidizing bacterium, *Ralstonia eutropha*. Considering the efficiency of utilization of a gas mixture as substrate, a practical fermentation process using *R. eutropha* for the mass production of P(3HB) from CO<sub>2</sub> should be designed on the basis of a recycled-gas, closed-circuit culture system. Also, maintaining the O<sub>2</sub> concentration in a gas phase lower than 6.9% (v/v) is essential to prevent the gas mixture from exploding. Our study, using an explosion-proof fermentation bench plant and a two-stage culture system with a newly designed air-lift fermenter, demonstrated that very high P(3HB) yield and productivity could be obtained while the O<sub>2</sub> concentration was maintained below 6.9%. However, a study on the continuous production of P(3HB) from CO<sub>2</sub> by chemostat culture of *R. eutropha* revealed that the productivity and content of P(3HB) in the cells was considerably lower than by fed-batch culture. It is deduced that the use of the hydrogen-oxidizing bacterium, *Alcaligenes latus*, which accumulates P(3HB) even in the exponential growth phase, will be useful for the effective production of P(3HB) from CO<sub>2</sub>.

### Introduction to hydrogen-oxidizing bacteria and biodegradable plastics

A hydrogen-oxidizing bacterium is a chemolithoautotrophic bacterium that can grow using a mixture of H<sub>2</sub>, O<sub>2</sub> and CO<sub>2</sub> gasses as substrate. This chemolithoautotrophic bacterium requires only inorganic salts like ammonium sulfate as nutrients in the culture medium. Hydrogen-oxidizing bacteria obtain energy for CO<sub>2</sub> fixation by the oxidation of H<sub>2</sub> gas with O<sub>2</sub> and occur in the natural environment, especially in the soil, ocean, rivers and hot springs. Among hydrogen-oxidizing bacteria, *Ralstonia eutropha*, which was reclassified from *Alcaligenes eutrophus*, (Yabuuchi et al. 1995), is the best characterized species, because its growth rate is much higher than that of other chemolithoautotrophic bacteria. However, the highest specific growth rate ( $\mu=0.68\text{ h}^{-1}$ ) was observed in *Hydrogenobacter thermophilus*, which was isolated by Goto et al. (1977a). Many studies on the physiology and basic character of hydrogen-oxidizing bacteria were reported by Goto et al. (1977b), Nishihara et al. (1991), Repaske (1961, 1966), Repaske and Mayer (1976), Repaske and Repaske (1976), Schlegel (1989), Schlegel and Lafferty (1971) and Schlegel et al. (1961). Biotechnological studies on autotrophic cultivation of hydrogen-oxidizing bacteria were mainly carried out by Foster and Litchfield (1964), Heinzle and Lafferty (1980), Kodama et al. (1975b, 1976), Miura et al. (1981, 1982), Ohi et al. (1979a, b), Schuster and Schlegel (1967) and Sonneleitner et al. (1979).

It is known that many hydrogen-oxidizing bacteria, especially *R. eutropha*, accumulate poly-D-hydroxybutyric acid, P(3HB), as a storage material within the cell under nutrient-limited conditions. P(3HB) is a kind of bacterial polyhydroxyalkanoate (PHA) and has potential as a raw material for manufacturing biodegradable thermoplastics. The general formula of PHAs is  $[-O-CH(R)-CH_2-CO-]_n$ , where  $R=CH_3$  in P(3HB). Ordinary plastic, which is mainly manufactured from fossil fuel, is non-degradable and therefore it easily accumulates in the en-

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vironment. The amount of non-degradable plastics accumulating in the environment is estimated to be  $25 \times 10^6$  t year<sup>-1</sup> (Dawes 1990). PHA produced by *R. eutropha* and other bacteria has thermoplastic properties and is decomposed by micro-organisms in the soil, rivers and oceans. Thus, PHA can be a candidate for a biodegradable plastic. In the production of microbial polyester, biomass and/or CO<sub>2</sub> are used as the carbon sources; and this does not threaten the fossil fuel reserves or increase the CO<sub>2</sub> concentration in the atmosphere. CO<sub>2</sub> gas used as a carbon source for the production of PHA can be obtained by the separation of exhaust gasses from factories and/or thermal power stations with a pressure swing adsorption system. At present, it is expected that H<sub>2</sub> gas can be supplied from the process of refining naphtha or generated by electrical dissociation of H<sub>2</sub>O with the energy from nuclear power stations.

Useful reviews on the general features of PHAs, PHA-producer strains and the fermentation process have been reported by many researchers (Anderson and Dawes 1990; Barham et al. 1992; Bonthron et al. 1992; Brandl et al. 1990; Braunegg et al. 1998; Byrom 1994; Choi and Lee 1997, 1999a; Cox 1994; Doi 1990; Doi et al. 1994; Inoue and Yosie 1992; Lafferty et al. 1988; Lee 1996; Madison et al. 1999; Sasikala and Ramana 1996; Steinbuchel 1991; Steinbuchel and Fuchtenbusch 1998; Steinbuchel et al. 1992). Most of these works were carried out with heterotrophic cultures, using organic substrate such as sugars, methanol and/or organic acids; and the effective production of PHAs at high concentration with high productivity was attained only in fed-batch cultures (Ahn et al. 2000; Kim et al. 1994, 1996; Rhee et al. 1993; Ryu et al. 1997; Suzuki et al. 1986; Tsuge et al. 1999; Wang and Lee 1997a, b; Yamane et al. 1996). In contrast, attempts at producing PHA from CO<sub>2</sub> using hydrogen-oxidizing bacteria are still rare, because autotrophic culture of the bacteria is accompanied by many difficult problems.

### The problems in autotrophic culture of hydrogen-oxidizing bacteria

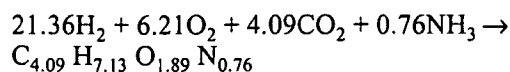
The substrate for autotrophic culture of hydrogen-oxidizing bacteria is a mixture of the gasses H<sub>2</sub>, O<sub>2</sub> and CO<sub>2</sub>. The composition of this gas mixture which attains sufficient cell growth in flask and plate cultures usually has a ratio of H<sub>2</sub>:O<sub>2</sub>:CO<sub>2</sub>=7:1:1. However, such a gas composition is completely within the gas-explosion range and therefore can easily explode. The low solubility of H<sub>2</sub> and O<sub>2</sub> gasses is another serious problem. Most of the studies on autotrophic culture of hydrogen-oxidizing bacteria have been carried out using conventional fermenters, through which the substrate gas is fed continuously and the exhaust gas is discharged outside the fermentation system. The discharge of the exhausted gas constitutes a loss in substrate and therefore the efficiency of gas utilization is decreased. To prevent the waste of substrate gas, two basic concepts for practical culture

system of hydrogen-oxidizing bacteria were suggested. One was the dead-end culture system by Bongers (1970) and the other was the recycled-gas culture system by Schlegel and Lafferty (1971). The dead-end culture system exhibits some disadvantages in gas mass-transfer, due to the lack of continuous feeding of substrate gas, while the recycled-gas, closed-circuit culture system attains high gas mass-transfer by recycling the substrate gas. Autotrophic cultivation of hydrogen-oxidizing bacteria using the recycled-gas culture system was only carried out by Kodama et al. (1975a), Repaske (1966) and the present authors, because the system was difficult to operate and needed a large gas reservoir. However, the authors obtained much biotechnological information on the autotrophic culture of *R. eutropha*, using the recycled culture system with a 200-ml jar fermenter (Ishizaki and Tanaka 1990a, b, 1991, 1992; Ishizaki et al. 1997; Sugimoto et al. 1999; Taga et al. 1997; Takeshita et al. 1993a, b; Tanaka and Ishizaki 1994; Tanaka et al. 1992, 1993, 1995).

### Autotrophic fermentation characteristics of *R. eutropha*

The features of cell growth and PHA accumulation in autotrophic culture of *R. eutropha*, which were demonstrated by the authors, are summarized as follows:

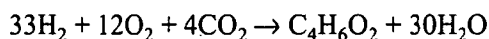
1. The specific growth rate of *R. eutropha* was considerably affected by the concentration of dissolved oxygen. The specific growth rate decreased with the increase in dissolved oxygen concentration. The maximum specific growth rate of 0.42 h<sup>-1</sup> was obtained at a concentration of dissolved oxygen of 5.0 kPa. *R. eutropha* could not grow at a dissolved oxygen concentration higher than 30 kPa (Ishizaki and Tanaka 1990a).
2. When the concentration of dissolved oxygen decreased below 3.2 kPa, oxygen became a limiting factor in the culture. Cell growth followed linear growth kinetics under oxygen-limited condition (Ishizaki and Tanaka 1990a, b).
3. Under oxygen-limited conditions, *R. eutropha* accumulated polyester in the cells, while the formation of cell protein and nucleic acids almost stopped. NMR analysis revealed that the polyester was the homopolymer of D-3-hydroxybutyric acid, P(3HB). In autotrophic culture, the production rate of P(3HB) by ammonium limitation, which is usually used as the best regime to promote P(3HB) accumulation, was lower than that by oxygen limitation (Ishizaki and Tanaka 1991).
4. The stoichiometric formulae for the formation of exponentially growing cells and P(3HB) were determined to be as follows (Ishizaki and Tanaka 1990a, 1991) for cell mass:



**Table 1** List of methods for the autotrophic, high-density cultivation of hydrogen-oxidizing bacteria

Strain	Culture method	Cell concentration (g l <sup>-1</sup> ·h <sup>-1</sup> )	Cell productivity (g l <sup>-1</sup> ·h <sup>-1</sup> )	Reference
<i>Ralstonia eutropha</i>	Batch	25.0	1.00	Repask and Meyer 1976
<i>Pseudomonas hydrogenovora</i>	Batch	24.0	0.50	Goto et al. 1977b
<i>Ralstonia eutropha</i>	Continuous	—	0.40	Morinaga et al. 1978
<i>Pseudomonas hydrogenothermophila</i>	Continuous	—	3.00	Igarashi 1986
<i>Alcaligenes hydrogenophilus</i>	Continuous	—	0.33	Miura et al. 1982

and for P(3HB):



- The amount of H<sub>2</sub> required for the fixation of CO<sub>2</sub> during the P(3HB) accumulation phase was 1.5 times larger than that during the exponential growth phase. However, according to the result of the analysis of ATP pool level in the cells, it was deduced that the ATP yield for the oxidation of 1 mol of H<sub>2</sub> was almost the same in both culture phases. It was suspected that the increase in the consumption of H<sub>2</sub> during P(3HB) accumulation under oxygen-limited condition was due to a decrease in the efficiency of ATP utilization in P(3HB) synthesis (Tanaka et al. 1992).
- The relationship between the overall volumetric coefficient of mass transfer for hydrogen, (K<sub>L</sub>a)<sub>H<sub>2</sub></sub>, and that for oxygen, (K<sub>L</sub>a)<sub>O<sub>2</sub></sub>, was expressed by the following formula:

$$(K_L a)_{\text{H}_2} = 0.280(K_L a)_{\text{O}_2}^{1.29}$$

The critical concentration of dissolved hydrogen for *R. eutropha* was also determined to be 11.6 kPa, using an on-line dissolved-hydrogen sensor (Takeshita et al. 1993a, b).

The biotechnological information described above was essential for designing a practical culture system of hydrogen-oxidizing bacteria to attain a high cell density with high cell productivity for the mass production of P(3HB) from CO<sub>2</sub>.

### Strategy to ensure high-density culture and to prevent the gas mixture from exploding

In autotrophic cultivation of hydrogen-oxidizing bacteria, it is not easy to attain a high cell concentration and/or high cell productivity, because the substrate gas mixture is insoluble in the culture medium and is very explosive. As shown in Table 1, in only a few cases were relatively high concentrations and productivities of cells obtained. In the autotrophic culture of hydrogen-oxidizing bacteria, the productivity of cell mass depends on the mass transfer rate of substrate gasses in the culture medium. Especially, P(3HB) accumulation is remarkably inhibited if dissolved hydrogen in the culture system is limited (Ishizaki and Tanaka 1991). Only an improvement in the coefficient of gas-mass transfer of the fermenter can enable an increase in the yield and productiv-

ity of cell mass. The improvement of gas-mass transfer is particularly important in the fermentation process to prevent the gas mixture from exploding. For a practical application of the autotrophic cultivation of hydrogen-oxidizing bacteria for the production of P(3HB) from CO<sub>2</sub>, it is absolutely essential to eliminate the potential of gas explosion. The strategy to prevent explosion is to maintain the oxygen concentration in the gas phase below the lower limit for explosion [6.9% (v/v)]. However, in such a low oxygen concentration, the productivity of the cell mass remarkably decreases. Therefore, a fermenter attaining a high value of K<sub>L</sub>a is essential for the effective production of PHA from CO<sub>2</sub>, employing hydrogen-oxidizing bacteria.

The work to prevent gas explosion in an autotrophic high-cell-density culture of hydrogen-oxidizing bacteria for the production of P(3HB) from CO<sub>2</sub> was mainly carried out by Tanaka and his colleagues (Sugimoto et al. 1999; Taga et al. 1997; Tanaka et al. 1993, 1995). They designed an explosion-proof fermentation bench-plant which was installed with several security devices and countermeasures; and they investigated P(3HB) production using *R. eutropha*, while maintaining the oxygen concentration in the gas phase below the lower limit for explosion [6.9% (v/v)]. A fermenter equipped with a specially designed, doughnut-shaped agitation system (K<sub>L</sub>a=2,970 h<sup>-1</sup>) was used to obtain a high oxygen transfer rate at such a low oxygen concentration. They obtained 91.3 g cells l<sup>-1</sup> and 61.9 g 3HB·l<sup>-1</sup> after 40 h of cultivation, while the oxygen concentration in the gas phase was maintained below 6.0% (v/v). The overall productivities of cells and P(3HB) were 2.28 g l<sup>-1</sup>·h<sup>-1</sup> and 1.55 g l<sup>-1</sup>·h<sup>-1</sup>, respectively.

Since a very high performance agitation system was essential in the fermentation plant, the authors developed a new culture method to obtain a relatively high productivity of cells and P(3HB), using a conventional jar fermenter (K<sub>L</sub>a=340 h<sup>-1</sup>). The new culture method was named the two-stage culture method (Tanaka and Ishizaki 1994). In this culture method, *R. eutropha* was heterotrophically grown in a fructose/mineral medium with aeration; and the cells were then cultivated in an inorganic medium for autotrophic P(3HB) accumulation by feeding the gas mixture with an oxygen concentration below 6.9%. Using 10, 20 and 30 g l<sup>-1</sup> fructose medium in the first stage, an average P(3HB) productivity of 0.56 g l<sup>-1</sup>·h<sup>-1</sup> was obtained in the autotrophic stage. The P(3HB) contents in the cells were 55.7–82.1% (w/w).

## P(3HB) production from CO<sub>2</sub> by chemostat culture

Continuous culture of hydrogen-oxidizing bacteria in autotrophic conditions were reported by several researchers (Ammann et al. 1968; Miura et al. 1982; Nishimura et al. 1991), although they were not focused on the production of P(3HB) but cell protein. In the study of an autotrophic chemostat culture of *R. eutropha* carried out by Morinaga et al. (1978), cell productivity was 0.2 g l<sup>-1</sup>·h<sup>-1</sup> and the percentage of P(3HB) in the cells was 20% (w/w) under oxygen-limited conditions. According to the report by Siegel and Ollis (1984), the P(3HB) content in the cells in autotrophic chemostat culture was 20% (w/w) at maximum.

We also investigated the continuous production of P(3HB) from CO<sub>2</sub> by two-stage culture, using an air-lift fermenter in the autotrophic stage (Taga 1996). An air-lift fermenter does not require mechanical agitation; and thus the energy consumption is lower than that of a stirred-tank fermenter. In this culture method, heterotrophic chemostat culture in the first stage was carried out by feeding fructose medium at a working volume of 100 ml and at the dilution rate of 0.1 h<sup>-1</sup>. The culture broth from the jar fermenter was transferred into the air-lift fermenter by a micro-tube pump; and chemostat culture was carried out for autotrophic P(3HB) accumulation. The working volume in the air-lift fermenter was 1.0 l and the dilution rate in the second stage was then 0.01 h<sup>-1</sup>. When 10 g fructose medium l<sup>-1</sup> was fed in the first stage, the P(3HB) content in the cells reached 57.6% (w/w). However, the highest P(3HB) productivity was only 0.025 g l<sup>-1</sup>·h<sup>-1</sup>. The lower productivity in chemostat culture, compared to the batch culture, was thought to be due to the limited mass transfer of oxygen in the second stage (the value for  $K_La$  of the air-lift type fermenter was determined to be only 100 h<sup>-1</sup>).

Hence, we used a newly designed air-lift fermenter with a draft tube and an HPLC filter as sparger; and we added a slight amount of carboxymethylcellulose (CMC) to culture medium to increase the  $K_La$  (Taga et al. 1997). Some kinds of surface-active reagents are known to increase the oxygen transfer rate (Bailey and Ollis 1986; Pirt 1975). Many researchers have reported that

the gas hold-up increases in the presence of CMC in the air-lift fermenter (Deckwer and Schumpe 1993; Kennard and Janekeh 1991; Schumpe and Deckwer 1982). The rheological change in the culture liquid in air-lift fermenters after adding surface-active reagents like CMC affects bubble formation, flow pattern and the mass transfer coefficient of oxygen (Kennard and Janekeh 1991). In our study, the  $K_La$  value of the air-lift fermenter, which was measured by a static method, increased to 375 h<sup>-1</sup> by the addition of 0.05% (w/w) CMC to the culture medium, while the  $K_La$  value without CMC addition was ca. 250 h<sup>-1</sup>. Actually, after the addition of 0.05% (w/w) CMC into the culture medium, the concentration and productivity of P(3HB) increased to 56.4 g l<sup>-1</sup> and 1.02 g l<sup>-1</sup>·h<sup>-1</sup>, respectively. The content of P(3HB) in the cells increased to 81.4% (w/w). The fermentation result was superior than those of any previous reports for autotrophic P(3HB) production using conventional stirred-tank fermenters (Ishizaki and Tanaka 1991; Morinaga et al. 1978; Siegel and Ollis 1984; Tanaka and Ishizaki 1994; Vollbrecht et al. 1979).

## Conclusion

Many attempts at the effective production of PHA with very high yield, coupled with high productivity, from organic carbon sources have been attained using only fed-batch culture. In most of the studies on continuous culture, the productivity and yield of PHA were found to be much lower than the values for fed-batch culture. As shown in Tables 2 and 3, the highest productivity of PHA obtained by heterotrophic continuous culture is about 1.0 g l<sup>-1</sup>·h<sup>-1</sup>, but the value is considerably lower than those obtained by fed-batch cultures. Also, in continuous autotrophic culture by our two-stage method, the productivity of P(3HB) was about 1.0 g l<sup>-1</sup>·h<sup>-1</sup>. In continuous cultures, which were carried out without regard to autotrophic or heterotrophic conditions, the productivity of PHA was lower than that in fed-batch culture. The reason why the productivity in continuous culture was lower than in fed-batch culture can be explained by the mechanism of accumulation of PHA in

**Table 2** Polyhydroxyalkanoate (PHA) production from organic carbon sources by heterotrophic continuous culture. *3HD* D-3-Hydroxydecanoate, *3HDD* D-3-hydroxyhexadecanoates, *3HDDE* D-3-

hydroxydodecenoate, *3HHx* D-3-hydroxyhexanoate, *3HO* D-3-hydroxyoctanoate, *3HTDE* D-3-hydroxytetradecanoate, *3HV* D-3-hydroxyvalerate

Bacterium used	Carbon source	Type of PHA	PHA productivity (g l <sup>-1</sup> ·h <sup>-1</sup> )	Reference
<i>Ralstonia eutropha</i>	Fructose and petanonic acid	3HV-co-3HV copolymer	0.31	Koyama and Doi 1995
<i>Pseudomonas putida</i> KT2442	Long chain fatty acids	3HHx-co-3HO-co-3HD-co-3HDD-co-3HDDE-co-3HTDE	0.69	Huiberts and Eggink 1996
<i>P. oleovorans</i>	n-Octane	Copolymer of 3-hydroxyalkanoates	0.58	Preusting et al. 1993
<i>P. oleovorans</i>	n-Octane	Medium-chain-length copolymer	1.06	Jung et al. 2001
<i>P. oleovorans</i>	n-Octane	Long-side-chain copolymer	0.18	Ramsay et al. 1991
<i>R. eutropha</i>	Lactate	P(3HB)	0.38	Henderson and Jones 1997

**Table 3** PHA production from organic carbon sources by heterotrophic fed-batch culture

Bacterium used	Type of PHA	PHA yield (g l <sup>-1</sup> )	PHA productivity (g l <sup>-1</sup> h <sup>-1</sup> )	Reference
<i>Protomonas extroquens</i>	P(3HB)	136.0	0.78	Suzuki et al. 1986
<i>R. eutropha</i>	P(3HB)	121.0	2.42	Kim et al. 1994
<i>R. eutropha</i>	3HB-co-3HV copolymer	90.4	1.81	Lee et al. 1995
Recombinant <i>Escherichia coli</i>	P(3HB)	98.7	4.94	Wang et al. 1997b
<i>Alcaligenes latus</i>	P(3HB)	68.4	4.00	Yamane et al. 1996
Recombinant <i>E. coli</i>	3HB-co-3HV copolymer	158.8	2.88	Choi and Lee 1999b

the cells: Most of the PHA-producing bacteria accumulate PHA under growth-limited conditions, due to a shortage of some nutrient, but hardly accumulate any PHA in the exponential growth phase. Therefore, in continuous culture, a high PHA content in the cells can be obtained only at a very low dilution rate, resulting in a low PHA productivity. Ramsay et al. (1990) pointed out that *A. latus* would be suited for polymer production in single-stage continuous culture, because it accumulates PHA in the cell to a high content, even during the exponential growth phase. This bacterium is also a hydrogen-oxidizing bacterium. If *A. latus* is used as the producer strain for P(3HB) production from CO<sub>2</sub> by chemostat culture, the productivity will be further improved at the higher dilution rate, with higher P(3HB) content. However, information on the growth characteristics of *A. latus* in autotrophic culture condition is very limited (the growth rate is thought to be apparently lower than that of *R. eutropha*). The biotechnological investigation on autotrophic culture of *A. latus* for PHA production from CO<sub>2</sub> will be a future task.

Although the cost of polyolefins is less than U.S. \$1 kg<sup>-1</sup> (Poirier et al. 1995), the price of PHA is much higher than that of synthetic plastics. However, recent calculations suggest that the price of PHA will be further reduced by an advanced fermentation strategy. Lee et al. (1999) reported that P(3HB) and medium-chain-length PHA can be produced at a cost of ca. U.S. \$2 kg<sup>-1</sup> by an effective production strategy to attain a productivity greater than 4 g l<sup>-1</sup> at a content of 80% of dry cell weight with a cheap carbon source. The improvement of the polyester content in the cell and productivity is the key for the practical application of P(3HB) production from CO<sub>2</sub>. Industrial applications for *A. latus* and other hydrogen-oxidizing bacteria for PHA production from CO<sub>2</sub> are expected in the near future.

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Cloning of the *Alcaligenes latus* Polyhydroxyalkanoate Biosynthesis Genes and Use of These Genes for Enhanced Production of Poly(3-hydroxybutyrate) in *Escherichia coli*

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Polyhydroxyalkanoates (PHAs) are microbial polyesters that can be used as completely biodegradable polymers, but the high production cost prevents their use in a wide range of applications. Recombinant *Escherichia coli* strains harboring the *Ralstonia eutropha* PHA biosynthesis genes have been reported to have several advantages as PHA producers compared with wild-type PHA-producing bacteria. However, the PHA productivity (amount of PHA produced per unit volume per unit time) obtained with these recombinant *E. coli* strains has been lower than that obtained with the wild-type bacterium *Alcaligenes latus*. To endow the potentially superior PHA biosynthetic machinery to *E. coli*, we cloned the PHA biosynthesis genes from *A. latus*. The three PHA biosynthesis genes formed an operon with the order PHA synthase,  $\beta$ -ketothiolase, and reductase genes and were constitutively expressed from the natural promoter in *E. coli*. Recombinant *E. coli* strains harboring the *A. latus* PHA biosynthesis genes accumulated poly(3-hydroxybutyrate) (PHB), a model PHA product, more efficiently than those harboring the *R. eutropha* genes. With a pH-stat fed-batch culture of recombinant *E. coli* harboring a stable plasmid containing the *A. latus* PHA biosynthesis genes, final cell and PHB concentrations of 194.1 and 141.6 g/liter, respectively, were obtained, resulting in a high productivity of 4.63 g of PHB/liter/h. This improvement should allow recombinant *E. coli* to be used for the production of PHB with a high level of economic competitiveness.

Recently, problems concerning the global environment have created much interest in the development of biodegradable polymers. Polyhydroxyalkanoates (PHAs) are polyesters of hydroxyalkanoates that are synthesized and intracellularly accumulated as an energy and/or carbon storage material by numerous microorganisms (1, 5, 15, 28). PHAs are considered to be good candidates for biodegradable plastics and elastomers since they possess material properties similar to those of synthetic polymers currently in use and are completely biodegradable after disposal (9).

A major problem in the commercialization of PHAs in a wide range of applications is their high production cost (3, 4). Much effort has been devoted to lowering the production cost by developing more efficient fermentation and recovery processes (15, 16, 28). Poly(3-hydroxybutyrate) (PHB) is the best known member of the PHAs and has been studied most often as a model product in the development of fermentation strategies.

To understand the mechanisms of PHA biosynthesis, studies on the metabolic pathways for PHA biosynthesis and molecular analyses of PHA biosynthesis genes in various bacteria have been conducted. In *Ralstonia eutropha* (formerly known as *Alcaligenes eutrophus*), acetyl coenzyme A is converted to PHB in three enzymatic steps (1, 35). A biosynthetic  $\beta$ -ketothiolase catalyzes the formation of a carbon-carbon bond by biological Claisen condensation of two acetyl coenzyme A moieties. An NADPH-dependent acetoacetyl coenzyme A (acetoacetyl-

CoA) reductase catalyzes the stereoselective reduction of acetoacetyl-CoA to D-(-)-3-hydroxybutyryl coenzyme A. The third reaction of this pathway is catalyzed by a PHA synthase, which links the D-(-)-3-hydroxybutyryl coenzyme A to the growing chain of PHB by an ester bond. After the first cloning of the PHA biosynthesis genes from *R. eutropha* (26, 32, 34), more than 30 different PHA biosynthesis genes were cloned from various bacteria (15). Cloning of various PHA biosynthesis genes not only has provided detailed information on the structure and organization of the PHA biosynthesis genes but also has allowed the creation of genetically engineered microorganisms or even plants for more efficient production of these biodegradable polymers and for the production of novel PHAs (16, 28).

One of the major factors affecting the overall production cost is productivity, defined as the amount of PHB produced per unit volume per unit time. *R. eutropha* and *Alcaligenes latus* have been used most often for the production of PHB, since PHB could be produced to a high concentration with high productivity (29, 39). Recombinant *Escherichia coli* strains harboring the *R. eutropha* PHA biosynthesis genes have also been used for the production of PHB (19, 22). A PHB concentration of as high as 157 g/liter could be obtained with a pH-stat fed-batch culture (40). Recombinant *E. coli* has been considered a strong candidate as a PHB producer due to several advantages over wild-type PHB producers, such as a wide range of utilizable carbon sources, PHB accumulation to a high content (up to 90% of cell dry weight), fragility of cells allowing easy recovery of PHB, and no degradation of PHB during fermentation due to the lack of intracellular depolymerases (6, 17). However, the highest productivity obtained with recombinant *E. coli* was 3.4 g of PHB/liter/h (40), considerably lower than that obtained with *A. latus* (4.94 g of PHB/liter/h) (39).

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## Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration

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**Abstract** With industrial development growing rapidly, there is a need for environmentally sustainable energy sources. Bioethanol (ethanol from biomass) is an attractive, sustainable energy source to fuel transportation. Based on the premise that fuel bioethanol can contribute to a cleaner environment and with the implementation of environmental protection laws in many countries, demand for this fuel is increasing. Efficient ethanol production processes and cheap substrates are needed. Current ethanol production processes using crops such as sugar cane and corn are well-established; however, utilization of a cheaper substrate such as lignocellulose could make bioethanol more competitive with fossil fuel. The processing and utilization of this substrate is complex, differing in many aspects from crop-based ethanol production. One important requirement is an efficient microorganism able to ferment a variety of sugars (pentoses, and hexoses) as well as to tolerate stress conditions. Through metabolic engineering, bacterial and yeast strains have been constructed which feature traits that are advantageous for ethanol production using lignocellulose sugars. After several rounds of modification/evaluation/modification, three main microbial platforms, *Saccharomyces cerevisiae*, *Zymomonas mobilis*, and *Escherichia coli*, have emerged and they have performed well in pilot studies. While there are ongoing efforts to further enhance their properties, improvement of the fermentation process is just one of several factors that needs to be fully optimized and integrated to generate a competitive lignocellulose ethanol plant.

### Introduction

Ethanol has been known for a long time, being perhaps the oldest product obtained through traditional biotechnology. Its current applications include potable, chemical, and fuel ethanol. Cars fueled by ethanol were already planned by Henry Ford in the 1880s, when he designed early model Ts that ran on “farm ethanol” made from corn. Early in the twentieth century, however, petroleum-derived fuels (fossil fuels) began to appear and quickly dominated the market. Low prices persisted for several decades until the advent of the “oil crisis” in the 1970s. This crisis underlined the importance of alternative energy sources, ethanol among them, giving birth to initiatives such as the National Alcohol Program in Brazil and the “gasohol” program in the USA. As oil prices decreased over the following decades, so did interest in fuel bioethanol. During the last few decades, however, the excessive consumption of fossil fuels, particularly in large urban areas, has greatly contributed to generating high levels of pollution. As a step to solve this problem, the addition of ethanol to gasoline, which reduces emission of carbon monoxide and unburned hydrocarbons that form smog (Wyman 1996), has widely been enforced in recent years. The demand for bioethanol has therefore increased.

Mature technologies for ethanol production are crop-based, utilizing substrates such as sugar cane juice and cornstarch. Since the cost of raw materials can be as high as 40% of the bioethanol cost (von Sivers et al. 1994 and earlier references therein; Wyman 1999), recent efforts have concentrated on utilizing lignocellulose. This natural and potentially cheap and abundant polymer is found as agricultural waste (wheat straw, corn stalks, soybean residues, sugar cane bagasse), industrial waste (pulp and paper industry), forestry residues, municipal solid waste, etc. (Wiselogle et al. 1996). It has been estimated that lignocellulose accounts for about 50% of the biomass in the world (10–50 billion tons according to Claassen et al. 1999).

The lack of a microorganism able to ferment efficiently all sugars released by hydrolysis from lignocellulosic

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materials has been one of the main factors preventing utilization of lignocellulose. Thus, an obvious target in the field of metabolic engineering has been the tailoring of such a microorganism, combining advantageous traits from different microorganisms with classical procedures such as random mutagenesis.

This review compares a biomass-derived fuel (ethanol) to a fossil fuel (gasoline), summarizes the current status of bioethanol production, discusses the efforts to construct a suitable microorganism for lignocellulose fermentation, briefly describes a model process emphasizing the importance of process integration, and provides visions for the future prospects for this technology.

## Renewable and fossil fuels

It is estimated that vehicles in the USA alone consume about 450 billion liters of fuel per year (EIA 1998), which has a great impact on the environment. Bioethanol and biodiesel have emerged as alternatives for fossil fuels. In comparing both types of fuels, ethanol and gasoline, four main aspects can be relevant:

*Performance.* Ethanol has a higher octane (ability to resist compression) rating than gasoline, enabling combustion engines to run at a higher compression ratio and thus giving a superior net performance (Wyman 1996). Additionally, the vapor pressure of alcohol is greater and the heat of vaporization is higher than that of gasoline, which is primarily responsible for the increased power outputs using alcohol. However, 1 gallon (3.78 l) of pure ethanol, due to its oxygen content, has 33% less energy than gasoline (Kosaric 1996).

*Pollution levels.* The incomplete combustion of fossil fuels emits gases known to contribute to smog formation. Furthermore, the extraction, processing, and combustion of fossil fuels result in air, water, and soil pollution and are thus hazardous to the environment and to public health (OFD 1999). By using biomass-derived ethanol, a net reduction in the levels of carbon dioxide (the main greenhouse gas) could range from 60–90% relative to gasoline-consuming vehicles (Brown et al. 1998). Thus, by utilizing fuel ethanol, the CO<sub>2</sub> released by combustion of the fuel would be recycled through the photosynthetic process, without net increase in CO<sub>2</sub> levels. The combustion of neat ethanol has the disadvantage of increased emission of aldehydes, particularly acetaldehyde, which is two to four times lower in gasoline emissions (Wagner 1980).

*Oil exhaustion.* Exhaustion of fossil fuels raises serious concerns about energy sources, and the American Institute of Petroleum has estimated that the petroleum supply will be exhausted some time in the twenty-first century (although new wells are still found, the cost of recovering the existent wells is becoming increasingly high). Exploitation of coals and oil shale deposits,

which are expected to be adequate for centuries, has a negative impact on the environment (Glazer and Nikaido 1995).

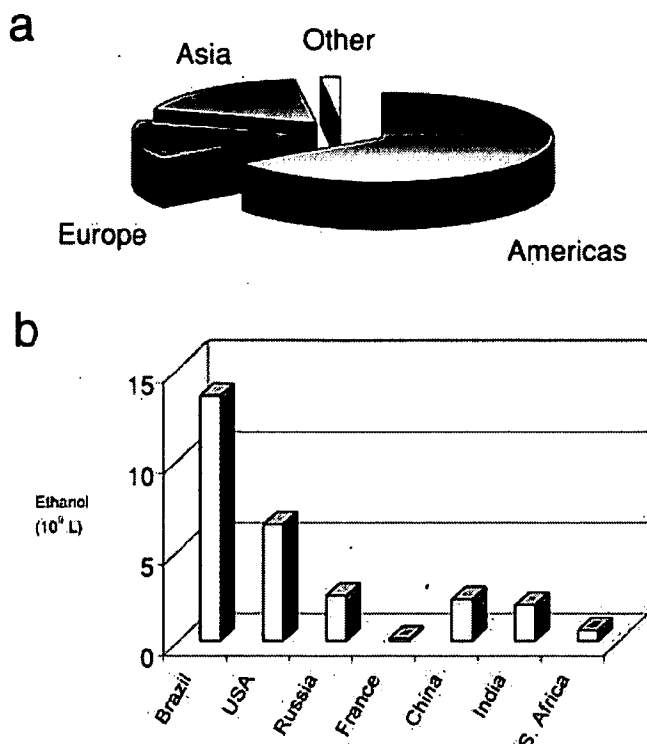
*Costs.* Wheals et al. (1999) have recognized the difficulty in obtaining data for a fair cost analysis. The direct costs for bioethanol production are higher than the production costs for gasoline. Thus, fuel ethanol enjoys governmental subsidies, as illustrated by the tax exemption (US\$0.11/l for the producer or blender) which will continue until 2007 in the USA. On the other hand, indirect costs for oil-derived fuels have been overlooked until recently, when a governmental agency in the USA recognized that “gasoline prices conceal huge, hidden indirect costs: although a nominal world oil price of US\$15, the indirect costs of gasoline with respect to air pollutant alone would be US\$ 45 per barrel and, furthermore, the cost of defending oil supplies in the Middle East could be as high as US\$9 per barrel” (Wheals et al. 1999). Additional spending is channeled into the Strategic Petroleum Reserve (SPR), which further increases the indirect costs (SPR is “the first line of defense against an interruption of supply”; DOE 1999). As of April 2000, the direct cost of 1 l of gasoline in the USA was US\$0.21 (in the rest of the world it was four- to five-fold higher) and the cost of 1 l of ethanol was US\$0.34 (RFA 1999).

## Ethanol production

In 1998, world total ethanol production was 31.2 billion liters (8.3 billion gallons) (Berg 1999). Only 7% of the total corresponded to synthetic alcohol (derived from gas or coal), the remainder being obtained by fermentation. Fuel ethanol accounts for approximately two-thirds of the total production. In terms of regional production, the Americas produced, in billions of liters, 20.3, Asia 5.5, Europe 4.7, Africa 0.5, and Oceania 0.2 (Fig. 1). Brazil was the largest producer in the world (13.5 billion liters, Fig. 1, inset); the USA attained 6.4 billion liters. Brazilian bioethanol was totally channeled into the fuel sector, whereas in the USA approximately 3.9 billion liters was used for the domestic fuel mix (Berg 1999).

In the European Union, more than 2 billion liters of ethanol were produced, but only 5% of this was used as fuel. Thirty percent of the production (120 million liters) corresponded to France, 18% to the United Kingdom, 17% to Germany, and 9% to Italy. Synthetic alcohol predominates in Germany and the United Kingdom. Sweden used 12 million liters of fuel ethanol, which corresponds to about 0.22% of the 5.5 billion liters of gasoline consumed (SPI 2000).

It is expected that the demand for ethanol for transportation will increase dramatically until 2010 (Bothast et al. 1999). In August 1999 an executive order was signed in the USA that specified the goal of tripling “biobased products and bioenergy” by the year 2010, which is projected to reduce the import of almost 4 bil-

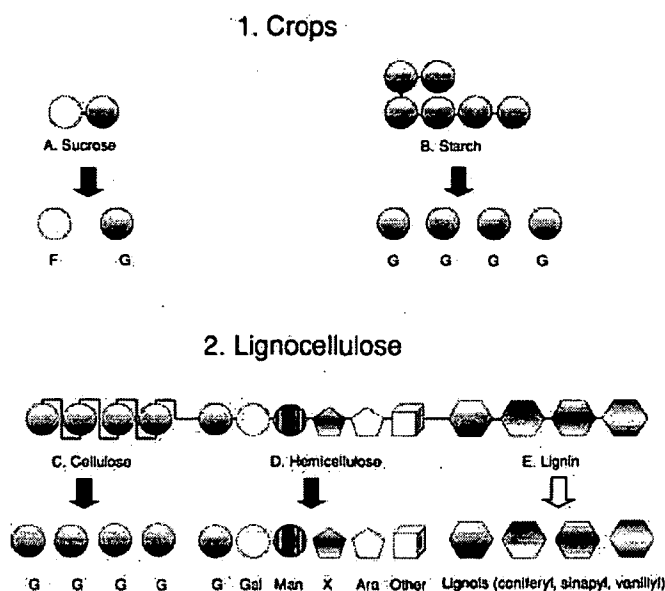


**Fig. 1** **a** Percentages of ethanol production by continent in 1998 (total production: 31.2 billion liters; Berg 1999). **b** The major ethanol producing countries

lion barrels of oil. Canada has made the environmental commitment to reduce emission of greenhouse gases to 6% below the 1990 levels by 2010. In Brazil, an increase in ethanol production is also expected, although at a slower pace than in previous years (Zanin et al. 2000). In the European Union, the commitment to lower CO<sub>2</sub> emission is expected to increase the use of renewable energy sources by 6% by 2010. With approval of a clean air law in France, the production of ethanol could increase to 500 million liters. In addition, there are ongoing projects of ethanol from biomass in the Netherlands, Sweden, and Spain (IEA 2000). In Sweden, a governmental goal is to have ethanol account for 15% of transportation fuel by the year 2010 (Mansson and Foo 1998).

### Raw materials for ethanol production

A variety of sources can provide sugars for bioethanol production, including crops and lignocellulose, as discussed below. The relevance of certain crops as raw material for ethanol production is indicated by the fact that over 90% of the world's bioethanol derives from crops (60% from cane sugar and beet sugar and the remainder from grains, mainly corn starch; Berg 1999). Crops such as sugar cane and sugar beet contain sucrose, which can be converted into its monomeric components (Fig. 2A); other crops, such as corn and cereals, contain starch, which can be converted into glucose (Fig. 2B). Sugar cane is the preferred raw material for ethanol production



**Fig. 2** Sources of sugars for ethanol production. **1** Crops; **2** lignocellulose. Arrows represent hydrolysis (only monomers generated from hydrolysis, represented by dark arrows, can be fermented). G Glucose, Gal galactose, F fructose, Man mannose, X xylose, Ara arabinose, Other L-rhamnose, L-fucose, uronic acids

in Brazil, India, and South Africa, whereas corn is used in the USA and sugar beet in France.

In contrast to sugar-containing crops, the utilization of lignocellulose as a substrate for ethanol production has a barrier in its complex structure, which resists degradation. Lignocellulose is composed of three main fractions: cellulose (~45% of dry weight), hemicellulose (~30% of dry weight), and lignin (~25% of dry weight) (Wiselogel et al. 1996).

*Cellulose*, the most abundant polymer on earth, is composed of thousands of molecules of anhydroglucose linked by  $\beta(1,4)$ -glycosidic bonds. The basic repeating unit is the disaccharide cellobiose (Fig. 2C). The secondary and tertiary conformation of cellulose, as well as its close association with lignin, hemicellulose, starch, protein and mineral elements, makes cellulose a hydrolysis-resistant molecule. Cellulose can be hydrolyzed chemically by diluted or concentrated acid, or enzymatically.

*Hemicellulose* is a highly branched heteropolymer containing sugar residues such as hexoses (D-galactose, L-galactose, D-mannose, L-rhamnose, L-fucose), pentoses (D-xylose, L-arabinose), and uronic acids (D-glucuronic acid) (Fig. 2D). Hemicellulose is more easily hydrolyzed than cellulose (Brigham et al. 1996). The composition of hemicellulose will depend on the source of the raw material (Wiselogel et al. 1996).

*Lignin*, the most abundant aromatic polymer in nature, is a macromolecule of phenolic character, being the dehydration product of three monomeric alcohols (lignols), *trans-p*-coumaryl alcohol, *trans-p*-coniferyl alcohol, and *trans-p*-sinapyl alcohol, derived from *p*-cinnamic acid (Kirk et al. 1977; Fig. 2E).



not clearly understood, procedures such as mutagenesis and screening have proven helpful. As a result of such strategies, a variety of organisms displaying attractive features for fermentation of lignocellulosics have been engineered in the last two decades. Today, however, efforts are concentrated on the three most promising microbial platforms, *S. cerevisiae*, *Z. mobilis*, and *Escherichia coli*. In the following discussion, these three microorganisms will therefore be emphasized. Traits considered either essential or desirable for lignocellulose fermentation, as listed in Table 1, will be subsequently described.

## Essential traits

### Broad substrate-utilization range

The ability to utilize all sugars present in lignocellulose substrate is a prerequisite for the efficient production of ethanol from the raw material. Given the high ethanol yields on glucose (and sucrose) as well as the high ethanol tolerance of *S. cerevisiae* and *Z. mobilis*, an obvious approach was to expand their substrate utilization range, so that all monosaccharides in lignocellulosic materials are utilized.

The ethanologenic microorganism *S. cerevisiae* displays several of the traits listed in Table 1, such as high ethanol yield and productivity, high tolerance to ethanol, tolerance to process hardness, GRAS status, and tolerance to low pH. In anaerobiosis it converts 1 mol of glucose into 2 mol ethanol, which results in the net production of 2 mol ATP plus CO<sub>2</sub>, through the glycolytic pathway (Fig. 3B).

For the large-scale production of ethanol from crop sugars, fermentations with *S. cerevisiae* are normally carried out at pH 5.0 and at 30 °C. To enable this organism to ferment xylose, the primary pentose present in hemicellulose, three main strategies have been approached: first, the insertion of bacterial xylose isomerase genes; second, the insertion of pentose utilization genes from *P. stipitis*; and third, the improvement of xylulose consumption.

1. The insertion of a bacterial xylose isomerase gene (from *E. coli*, *Bacillus subtilis*, or *Thermos thermophilus*) did not allow for xylose fermentation. This was attributed to factors such as differences in internal pH between bacteria and yeast, unsuitable folding of the enzyme, unsuitable post-translational modifications, inter-intramolecular disulfide bridge formation, and the absence of essential cofactors or ions. (Sarthy et al. 1987; Amore et al. 1989). Post-translational modification was ruled out by Sarthy et al. (1987).
2. The insertion of the *P. stipitis* genes *XYL1* (xylose reductase) and *XYL2* (xylitol dehydrogenase) enabled *S. cerevisiae* to grow on xylose and produce low levels of ethanol; 1.6 g ethanol/l was produced from 21.7 g xylose/l (Kötter and Ciriacy 1993; Table 2). Neither

overexpression of *XYL1*+*XYL2* nor chromosomal integration of these genes improved xylose fermentation substantially (Tantirungkij et al. 1993; Table 2). Low ethanol yields have been attributed to: (a) xylitol production, (b) an inefficient PPP, and/or (c) the inability of pentose sugars to activate the lower part of glycolysis (Boles et al. 1993). Xylitol accumulation and excretion would be due to cofactor imbalance (NADPH/NAD<sup>+</sup>) in the first two reactions leading to xylose metabolism (Fig. 3A). An inefficient PPP would result from the imbalanced activities of enzymes such as transaldolases (TALs) and transketolases (TKLs) (Fig. 3A), resulting in accumulation of intermediates such as sedoheptulose-7-P, a substrate for transaldolase (Senac and Hahn-Hägerdal 1991). As glyceraldehyde-3-phosphate (glyceraldehyde-3-P), a key intermediate in glycolysis, can also be generated in the PPP, an inefficient PPP will affect the lower part of glycolysis by limiting the levels of glyceraldehyde-3-P and result in decreased levels of ethanol (Boles et al. 1993).

Therefore, to avoid the accumulation of sedoheptulose-7-P, a further step was the construction of strains able to express different ratios of XR and XDH, in addition to the overexpression of *TKL1* (encoding a transketolase) and *TAL1* (encoding a transaldolase) (Bao et al. 1997; Walfridsson et al. 1997). A strain with a XR:XDH activity ratio of 0.06 had higher xylose consumption (3.25 g/l xylose), no xylitol formation, low levels of glycerol and acetic acid formation, and produced more ethanol than the strain with a higher XR:XDH ratio, but it was still far from being economically significant.

3. The improvement of xylulose consumption was approached by Deng and Ho (1990), who demonstrated that phosphorylation is a vital step for metabolism of xylose through the PPP (Fig. 3B). The gene *XKS1* (encoding xylulokinase) from *S. cerevisiae* and the heterologous genes from *XYL1* and *XYL2* (from *P. stipitis*) were inserted into a hybrid host, obtained by classical breeding of *S. uvarum* and *S. diastaticus*, which resulted in *Saccharomyces* strain 1400 pLNH32, capable of growing on xylose alone. However, xylitol yield was still high, i.e., 0.1 g xylitol per g xylose, probably due to a cofactor imbalance in the XR and XDH. This strain fermented xylose at 66% of the theoretical yield (Moniruzzaman et al. 1997a; Table 2). In a mixture of sugars, 90% of the yield was achieved, but arabinose was not metabolized. In AFEX (ammonia fiber explosion, a detoxification procedure)-pretreated corn-fiber hydrolysate, containing minimal levels of inhibitory compounds, this strain attained a yield of 0.50 (g of ethanol per g sugar consumed) and a maximum volumetric productivity of 1.60 g/l per h (Moniruzzaman et al. 1997a).

Another constructed strain (*Saccharomyces* 1400 pLNH33), containing the three genes in a multicopy vector, was able to grow on glucose and xylose, but was unstable in non-selective media (Ho et al. 1993).

**Table 2** Performance of biocatalysts in the presence of sugars present in lignocellulose. *G* Glucose, *X* xylose, *A* arabinose, *Gal* galactose, *Man* mannose. The number specifies the initial sugar concentration in g/l. All fermentations (except Eliasson et al.

2000) were carried out as batch cultures and the specified concentration is the final (or maximum) ethanol concentration. Ethanol yield is given in percentage of the theoretical yield; for glucose and xylose it is 0.51 g ethanol/g glucose

Biocatalyst	Substrate	Ethanol (g/l)	Ethanol yield (% of theoretical)	Volumetric productivity (g/l per h)	References
<i>S. cerevisiae</i> (XYL1, XYL2)	X21.7	1.6	14.5	0.07	Kötter and Ciriacy (1993)
<i>S. cerevisiae</i> (XYL1, XYL2)	X50	2.7	10.6	0.02	Tantirungkij et al. (1993)
<i>Saccharomyces</i> 1400 (pLNH32)	X80	27.0	66	1.12	Moniruzzaman et al. (1997a)
<i>Saccharomyces</i> 1400 (pLNH32)	G31, X15, A10 <sup>a</sup> , Gal2	22.0	90	0.92	Moniruzzaman et al. (1997a)
<i>Saccharomyces</i> 1400 (pLNH32)	Corn fiber AFEX hydrolysate (G35, X7.5, A5, Gal1)	21.0	98	1.60	Moniruzzaman et al. (1997a)
<i>S. cerevisiae</i> TMB 3001 (XYL1, XYL2, XKS1) integrated	G5, X15 <sup>b</sup>	2.5	25	0.15	Eliasson et al. (2000)
<i>Z. mobilis</i> CP4 (pZB5)	X25	11.0	86	0.57	Zhang et al. (1995)
<i>Z. mobilis</i> CP4 (pZB5)	G25, X25	24.2	95	0.81	Zhang et al. (1995)
<i>Z. mobilis</i> ZM4 (pZB5)	G65, X65	62.0	90	1.29	Joachimsthal et al. (1999)
<i>Z. mobilis</i> ATCC 39676 (pZB186)	G25, A25	21.42	84	—	Deanda et al. (1996)
<i>Z. mobilis</i> ATCC 39676 (pZB301)	G30, X30, A20	33.5	82–84	0.82–0.65	Chou et al. (1997); Zhang et al. (1998, unpublished data)
<i>Z. mobilis</i> CP4 (pZB5)	Corn-fiber acid hydrolysate	22.6	88	1.04	Bothast et al. (1999)
<i>E. coli</i> KO11 ( <i>pdg</i> , <i>adhB</i> , <i>frd</i> -) integrated	X80	41.6	102 <sup>c</sup>	0.87	Ohta et al. (1991)
<i>E. coli</i> KO11	Pine-steam-pretreated, G20.0, X15.0, Gal5.0, Man30.7, A2.1	32.0	85	0.67	Barbosa et al. (1992)
<i>E. coli</i> KO11	Corn-fiber acid hydrolysate	34.7	80	1.16	Dien et al. (1997)
<i>E. coli</i> KO11	Corn-fiber AFEX hydrolysate	27.1	92	1.05	Moniruzzaman et al. (1997a)

<sup>a</sup> Not accounted in yield calculation

<sup>b</sup> Continuous culture,  $D=0.06\text{ h}^{-1}$  (specific productivity: 0.24 g/g DW/h)

<sup>c</sup> Attributed to nutrient supplement

Chromosomal integration resulted in high stability, and the resultant *Saccharomyces* 1400 LNH-ST grew well in a glucose-xylose mixture, attaining 70% of the theoretical yield in a two-stage continuous process (Toon et al. 1997). When grown in pretreated corn biomass, it produced 63.5% of the theoretical yield in a 9,000-l batch reactor, utilizing a variation of the simultaneous saccharification (hydrolysis) and fermentation process (see "Processes for bioethanol production", below) (Toon et al. 1997). By contrast, the chromosomal integration of a single copy of the *XYL1-XYL2-XKS1* cassette into *S. cerevisiae* resulted in strain TMB3001, which in minimal medium containing glucose and xylose in continuous culture attained specific uptake rates of 0.47 g/g per h and 0.21 g/g per h respectively, for glucose and xylose (Eliasson et al. 2000; Table 2).

A current approach for improving pentose utilization in *S. cerevisiae* is the insertion of genes for arabinose metabolism and xylose transport. For arabinose, good sources of genes are yeasts such as *Candida aurigiensis* (Jeffries and Shi 1999), and for xylose transport, *P. stipitis*. That fungi (including yeasts)

might be better gene sources for the metabolism of arabinose has also been recently suggested by Sedlak and Ho (2001). Indeed, these authors expressed the genes from the *araBAD* operon from *E. coli* in *S. cerevisiae*, but the transformed strain was not able to produce a detectable amount of ethanol from L-arabinose.

The second ethanologenic microorganism, *Z. mobilis*, employed in beverage production, ferments at pH 5.0 and at temperatures between 30 and 40 °C. It consumes glucose through the Entner- Doudoroff pathway (ED; Fig. 3C), in which 1 mol of glucose yields 2 mol of pyruvate, generating 1 mol of ATP. To remain competitive with this low energetic yield, its metabolism is greatly accelerated. Therefore, *Z. mobilis* possesses elevated levels of glycolytic and ethanologenic enzymes (pyruvate decarboxylase – PDC and alcohol dehydrogenase – ADH) resulting in outstanding ethanol yields (around 97% of theoretical; Zhang et al. 1995). In order to expand its substrate spectrum, which naturally includes sucrose and its derived monomers, strategies such as insertion of the genes for xylose and arabinose utilization have been applied.



1. In enabling xylose utilization, the insertion of *xylA* (xylose isomerase) and *xylB* (xylulokinase) from *Xanthomonas campestris* and *Klebsiella pneumoniae* resulted in *Z. mobilis* strains unable to grow on xylose as sole carbon source (Feldmann et al. 1992). Enzymatic assays indicated an inefficient activation of the PPP. Thus, both 6-phosphogluconate dehydrogenase (responsible for the formation of 2-KDPG, a precursor of glyceraldehyde-3-P and pyruvate; Fig. 3C) and TKL activities were low and TAL activity was null (Fig. 3A). To overcome this limitation, four genes from *E. coli*, *xylA* (xylose isomerase), *xylB* (xylulokinase), *tal* (transaldolase), and *tktA* (transketolase), were inserted in the host. The recombinant, *Z. mobilis* CP4 (pZB5), showed enzymatic activity for all four enzymes and grew on xylose as the sole carbon source, attaining 86% of the theoretical yield (Zhang et al. 1995; Table 2). In the fermentation of a sugar mixture, 95% of the theoretical yield was achieved with this strain in 30 h (Zhang et al. 1995; Table 2). *Z. mobilis* CP4 (pZB5) has also been grown in acid-pretreated corn fiber, and 88% of the theoretical yield was achieved, with a productivity of 1.04 g/l per h (Bothast et al. 1999; Table 2). More recently, the xylose utilization genes were integrated into the *Z. mobilis* chromosome and the genetic stability of the strain was improved (Zhang et al., unpublished results).
2. In enabling L-arabinose utilization, five genes from *E. coli*, *araA* (L-arabinose isomerase), *araB* (L-ribulokinase), *araD* (L-ribulose-5-phosphate-4-epimerase), *talB* (transaldolase), and *tktA* (transketolase), were inserted into the microorganism, which resulted in strain *Z. mobilis* ATCC 39676 (pZB186). With this strain, 98% of the theoretical yield was achieved in arabinose-based medium; furthermore, in a mixture of sugars (Table 2), the yield was 84% of the theoretical. Some arabinose was not consumed and glucose was preferentially utilized (Deanda et al. 1996).
3. One strain of *Z. mobilis* has been constructed with seven plasmid-borne genes encoding xylose- and arabinose-metabolizing genes and PPP genes. This strain, *Z. mobilis* 206C (PZB301), was capable of fermenting both xylose and arabinose. In a mixture of sugars, 82–84% of the theoretical yield was attained in 80–100 h at 30 °C (Chou et al. 1997; Zhang et al. 1998, unpublished results; Table 2).

### High ethanol yields and productivity

With ethanol being a low value-added product, the overall yield in the conversion of sugars to ethanol is pivotal. Utilizing crop sugars as substrates for ethanol production, yields of 90–95% of the theoretical can be obtained using *S. cerevisiae* or *Z. mobilis*, and yields in this range are also required for an economically feasible process based on lignocellulose as raw material. However, of equal importance to the yield is a high productivity,

since the depreciation of capital investments also contributes significantly to the cost of ethanol production. The overall productivity (often specified as g ethanol per l reactor per h) is difficult to compare between different reports, because it depends on both the amount of biomass and the activity of the biomass. It is therefore preferential to use the specific productivity (given as g ethanol per g dry weight per h), but this value is rarely reported in the literature, and this makes a direct comparison difficult. In order to have an economically feasible process, the overall productivity of ethanol has to be above 1.0 g ethanol/l per h, and this value can at least be used for evaluation of different strains. In Table 2, we have collected and reported values for the yield and productivity of ethanol for the three most developed microorganisms. *S. cerevisiae* and *Z. mobilis* were already discussed in the previous section.

*E. coli* and several enteric bacteria naturally possess a broad substrate-utilization range, converting hexoses (glucose, mannose, galactose, fructose), pentoses (xylose and arabinose), and uronic acids (galacturonic acid, glucuronic acid) to the central metabolite, pyruvate. This compound is further converted to a near equal mix of ethanol, lactate, acetate, and formate (H<sub>2</sub>O plus CO<sub>2</sub>). Normally, fermentations are carried out at pH 7.0 and at temperatures between 30 and 35 °C. The main strategy to increase ethanol production in *E. coli* and make it suitable for lignocellulose processes was to redirect the carbon flux towards ethanol production, which was achieved in three main steps.

- (1) The insertion of *pdh* and *adhB* genes from *Z. mobilis*, encoding for highly active ethanologenic enzymes, enabled *E. coli* to produce ethanol and CO<sub>2</sub> from hexoses and pentoses at high efficiency. Furthermore, *pdh* and *adhB* were placed under the control of a single promoter, creating the PET (production of ethanol) operon (Ingram et al. 1987).
- (2) The PET operon was subsequently introduced into several bacterial hosts. After thorough screening, *E. coli* strain ATCC 11303 (*E. coli* B) appeared to be the most hardy to environmental stress and it was chosen as the host for the chromosomal integration of the PET operon. Surprisingly, after integration, levels of *pdh* and *adhB* were far below those observed with pUC18-based constructs, and the redirection of pyruvate metabolism was inadequate (Ingram and Conway et al. 1988). Further selection by classic genetics methods was pursued based on the apparent linkage between expression of the *pdh* and *adhB* genes to that of the *cat* gene immediately downstream (Ohta et al. 1991). Hence, utilizing high levels of chloramphenicol (600 µg/ml) in solid media, large raised colonies appeared, which are indicative of high expression of the ethanologenic genes in *E. coli*, as reported by Conway et al. (1987). Moreover, when grown on special plates to detect activity of alcohol dehydrogenase, these colonies presented a dark-red phenotype, which facilitated

the visual screening for highly ethanologenic colonies (Conway et al. 1987). Resultant mutants presented desired levels of ethanologenic enzymes (Ohta et al. 1991). One of them, designated *E. coli* KO4, was further modified to eliminate succinate formation, as described in the following section, and was able to ferment the major sugars present in hemicellulose with ethanol yields higher than 90%.

- (3) The direction of carbon flux towards ethanol formation was favored by the expression of high levels of heterologous *pdc* and *adhB* as well as by the fact that the original PDC from *Z. mobilis* has an affinity towards pyruvate higher than other homologous enzymes competing for pyruvate in *E. coli*, e.g., lactate dehydrogenase. The performance of strain KO11 has been extensively tested (described by Olsson and Hahn-Hägerdal 1996; reviewed by Ingram et al. 1997, 1998). Hemicellulose syrups derived from a mixture of corn hulls and fiber were supplemented with yeast extract and tryptone, 5 g/l and 10 g/l, respectively. A yield efficiency of 88% (0.85 g/l per h, 0.45 g ethanol/g sugar consumed) was achieved in a hydrolysate containing 90 g sugars /l (Ingram et al. 1998). The utilization of a similar hydrolysate syrup, supplemented with corn steep liquor and containing 69 g total sugar /l, resulted in 35 g ethanol/l (0.73 g/l per h, 0.51 g/g) during growth in a 25-l reactor (Ingram et al. 1997). The successful fermentation of laboratory sugars by strain KO11 in 10,000-l scale has been reported (Ingram et al. 1997). Moreover, the fermentation capability of this strain in pine-steam-pretreated hydrolysate, corn-fiber acid hydrolysate, and AFEX-treated corn fiber has been investigated, respectively, by Barbosa et al. (1992), Dien et al. (1997), and Moniruzzaman et al. (1997a). As seen in Table 2, the ethanol yields were 85%, 80%, and 92%, respectively. From these and other similar tests, KO11 emerged as one of most efficient microorganisms currently available for the fermentation of a mixed sugars stream (Hahn-Hägerdal et al. 1994a; McMillan 1996; Chandrakant and Bisaria 1998; Bothast et al. 1999).

#### Minimal byproduct formation

A well-known byproduct in yeast fermentation is glycerol. During the formation of biomass, there is a net conversion of the cytosolic cofactor  $\text{NAD}^+$  to NADH. Since the respiratory chain is non-functional under anaerobic conditions, the only route to reconvert the cofactor to  $\text{NAD}^+$  is through glycerol formation. Thus, glyceraldehyde-3-P is converted to dihydroxyacetone-P to glycerol-3-P, and further to glycerol. There are two genes, *GPD1* and *GPD2*, encoding glyceraldehyde-3-phosphate dehydrogenase, the enzyme that regenerates  $\text{NAD}^+$  from NADH while converting dihydroxyacetone-P to glycerol-3-P, but *Gpd2* is the most important for glycerol formation (Nissen et al. 2000a).

Two successful attempts in lowering byproduct formation in ethanolic fermentation have been reported. In the first, a *GPD2*-mutant of *S. cerevisiae*, grown under anaerobic conditions had a 40% reduction in glycerol levels (relative to the amount of substrate consumed) and 8% higher ethanol yield than the unmodified strain, although the maximal specific growth rate was 45% lower than the latter (Nissen et al. 2000a). In a second approach to decrease glycerol formation, the redox metabolism was engineered by changing the cofactor requirements associated with ammonium assimilation. Glutamate dehydrogenase, a key enzyme linking carbohydrate and nitrogen metabolism, comprises isoenzymes encoded by the genes *GDH1* and *GDH2*. Glutamate dehydrogenase catalyzes the incorporation of  $\text{NH}_4^+$  into  $\alpha$ -ketoglutarate, which results in the formation of L-glutamate. *Gdh1* and *Gdh2* require NADPH and NADH, respectively, as cofactors. Since biomass formation generates a surplus of NADH, this cofactor could be redirected towards ammonium assimilation, provided that glutamate formation is restricted to the isozyme *Gdh2*, which consumes exclusively NADH. Therefore, the net accumulation of NADH during the process of biomass synthesis would decrease, and the need for re-oxidation of this cofactor through glycerol formation would, consequently, decrease. By applying this strategy, the production of NADH associated with biomass synthesis decreased significantly, resulting in a more than 40% reduction of the glycerol yield (Nissen et al. 2000a). Alternatively, *Gdh1* was replaced by the glutamate synthase-glutamine amide:2-oxoglutarate aminotransferase (GS-GOGAT) system, which also uses NADH as cofactor. In this pathway, there is also consumption of ATP, and besides the 40% reduction in glycerol yield there was an 8% increase in ethanol yields (Nissen et al. 2000b).

Succinate is another byproduct generated in ethanol production. In order to eliminate succinate formation in ethanologenic *E. coli* KO4 and consequently increase the ethanol yield, the fumarate reductase gene (*frd*) was deleted generating *E. coli* strain KO11. In this strain, the channeling of a small fraction of phosphoenolpyruvate towards the formation of succinate was avoided (Ohta et al. 1991). Nevertheless, eliminating competing pathways, for example, by deleting the genes *ldh* and *pfl*, whose products are responsible for the formation of lactic acid and formate from pyruvate, respectively, had little effect on the efficiency of ethanol production, provided that high levels of PDC were available (Ingram et al. 1991).

#### Increased ethanol tolerance

For low-value products such as ethanol, a product concentration as high as possible is essential for the process economy. What normally occurs is that, as the ethanol concentration in the broth increases, most microorganisms begin to experience some impairment of membrane integrity. According to Dombek and Ingram (1986), the

response to ethanol stress correlates with the type of lipids in the cellular membrane. In fact, the two well-known ethanologens, *S. cerevisiae* and *Z. mobilis*, display peculiar membrane structures. Thus, the membrane of *S. cerevisiae* is rich in sterols, whereas the membrane of *Z. mobilis* is exceptionally rich in the fatty acid *cis*-vaccenic acid, as well as in compounds known as hopanoids (analogous to sterols). *S. cerevisiae* tolerates up to 21% (w/v) ethanol (Walker 1998), whereas *Z. mobilis* tolerates up to 12% (w/v) ethanol (Rogers et al. 1996).

Besides the cell membrane composition, factors such as the activity of plasma membrane ATPase and superoxide dismutase, and the capacity of a strain to produce trehalose contribute to the ethanol tolerance trait in yeasts (Jeffries and Jin 2000). In *S. cerevisiae*, the plasma membrane (PM) proton pump  $H^+$ -ATPase is an abundant and essential enzyme (Monk et al. 1995), existing as isoforms Pma1 and Pma2, encoded by *Pma1* and *Pma2*, respectively (Supply et al. 1995). PM ( $H^+$ -ATPase) permits the coupling of ATP hydrolysis to proton extrusion, a cellular mechanism that allows both solute uptake by a secondary transporter and regulation of intracellular pH. The *in vivo* activation of *S. cerevisiae* plasma PM  $H^+$  ATPase by ethanol has been studied by Monteiro and Sá-Correia (1998). A stress-inducible regulator of ATPase is Hsp30, which is induced by exposure to ethanol as well as by heat shock, severe osmotic stress, weak organic acid exposure, and glucose limitation (Piper et al. 1997).

Perhaps the best example of increased microbial tolerance to ethanol was described in a report by Yomano et al. (1998). As a result of classic random mutation techniques, *E. coli* KO11 became more tolerant to ethanol. To select for the ability to grow on ethanol, strain KO11 was inoculated in broth containing 35 g ethanol/l. After 5, 13, and 14 sequential transfers, the ethanol concentration was increased to 40, 45, and 50 g/l, respectively. After three to four transfers, dilution and plating on solid media were interspersed to enrich for colonies that retained the ethanol production trait (Conway et al. 1987), i.e., large raised colonies indicating high ethanol production. (Dilution into ethanol concentrations higher than 50 g/l did not yield mutants with a further increase in ethanol tolerance that retained both rapid growth and ethanol production traits). Colonies were maintained in 50 g ethanol/l for 3 months before they were selected. Twenty clones were tested for ethanol production in pH-controlled fermentors. The best was designated *E. coli* LY01, which in xylose-containing broth achieved 85% of the ethanol theoretical yield after 72 h and reached a final concentration of over 60 g/l (7.5% (v/v) by volume); with glucose, 96 h was required to achieve a similar yield.

#### *Increased tolerance to inhibitors*

To facilitate the release of sugars from the hemicellulose and cellulose fractions of lignocellulose, hydrolysis is

necessary. Given the complex structure of lignocellulose, such treatments can be harsh, requiring high temperatures and/or the addition of chemicals. By applying such procedures, other compounds will be produced in addition to sugars, which can affect lignocellulose fermentation depending on the sensitivity of available biocatalysts to these inhibitors. The levels of inhibitors present in the syrup depend on factors such as the lignocellulose source, amount of solids in the reactor, and the pretreatment condition: time, pH, temperature, concentration of chemicals, extent of water recirculating in the process, etc. (Clark and Mackie 1984; Buchert et al. 1990; Buchert and Niemella 1991; Palmqvist et al. 1996; Palmqvist and Hahn-Hägerdal 2000a, 2000b); Larsson et al. 1997; Larsson et al. 1999a; Taherzadeh et al. 1997a; Stenberg et al. 1998a). Such inhibitory compounds in hydrolysates can derive from: (1) sugar degradation (furfural from pentoses, hydroxymethylfurfural from hexoses); (2) pretreatment release (acetic acid, formic acid); (3) lignin degradation (lignols and other phenolic alcohols, acids and aldehydes), and (4) the pretreatment vessel and/or woody biomass (inorganics; Fengel and Wegener 1984; Ranatunga et al. 2000).

Inhibitors affect the overall cell physiology and often result in decreased viability, ethanol yields, and productivity. In *S. cerevisiae*, the inhibition of fermentation by furans (Palmqvist et al. 1999a, b; Taherzadeh et al. 2000), aliphatic acids (Taherzadeh et al. 1997b; Palmqvist et al. 1999b) and aromatic compounds (Ando et al. 1986; Larsson et al. 2000) has been studied. In *Z. mobilis* CP4 (pZB5), several aliphatic and aromatic inhibitory compounds were studied (Ranatunga et al. 1997). In ethanologenic *E. coli* KO11 and LY01 similar studies were carried out (Zaldivar and Ingram 1999; Zaldivar et al. 1999; Zaldivar et al. 2000). In these studies, the tolerance of *E. coli* towards inhibitors was investigated. Twenty-two compounds were grouped into acids (acetic, ferulic, vanillic, etc.), aldehydes (furfural, hydroxymethylfurfural, syringaldehyde, etc.) and alcohol compounds (coniferyl, vanillyl, guaiacol, etc.) and their effect on growth and ethanol production were examined. These studies concluded that: (a) the toxicity in hydrolysate is determined by the aggregate effect of compounds, rather than by one specific compound; (b) furfural affects central carbon metabolism and, in combination with other aldehydes, acids, and alcohols, including ethanol, has a synergistic, i.e., more than additive, effect; (c) aromatic compounds are more toxic than furans; (d) toxicity correlates with hydrophobicity; (e) strain LY01 is more tolerant to inhibitors than its parental strain KO11. Consequently, the suitability for a certain process organism will depend on the current process situation, in which pretreatment, hydrolysis, and the degree of recirculation of the streams will determine the conditions under which the process organism will work.

The mechanism/s underlying inhibition are different and depend on the chemical structure of the inhibitor. For instance, furfural inhibits glycolytic enzymes *in vitro* (Banerjee et al. 1981b) and has an additional effect on

aldehyde dehydrogenase activity, resulting in an accumulation of acetaldehyde that would be responsible for the lag-phase during the growth of *S. cerevisiae* in the presence of this furan (Palmqvist et al. 1999a). Furfural (4 g/l) severely decreases the specific growth rate and it can be reduced to the less inhibitory furfuryl alcohol (Taherzadeh et al. 1999a); moreover, the conversion to furfuryl alcohol affects the intracellular redox balance (Palmqvist et al. 1999a). Hydroxymethylfurfural is less inhibiting than furfural, but remains in the medium about four times longer due to its low conversion rate (Taherzadeh et al. 2000).

For the toxicity of weak acids, two mechanisms, uncoupling and intracellular anion accumulation, have been proposed (Russell 1992). Thus in acidic pH, the conjugate-neutral form of the acid crosses the cell membrane and at a higher intracellular pH, normally neutral, it dissociates, causing protons (and anions) to accumulate. As a result, intracellular pH decreases collapsing  $\Delta$ pH, important for energy generation and other cellular processes. There have been indications, however, that the accumulation of the anionic form of the acid, rather than the uncoupling effect, is more important for the toxicity of weak acids (Russell 1992). Thus, when *S. cerevisiae* was grown on glucose under anaerobic conditions, in the absence of added acetic acid, growth was possible at pH 2.5, whereas a total acetic acid addition of 10 g/l increased the minimum allowable pH to 4.5; moreover, it was shown that the concentration of the undissociated form of acetic acid should not exceed 5 g/l for growth to occur (Taherzadeh et al. 1997b). In the case of hydrophobic inhibitors, they would target hydrophobic sites such as multiprotein complexes, membrane transport systems, and the hydrophobic core of nucleic acids (Dombek and Ingram 1986; Isken and de Bont 1998).

The ability to degrade inhibitors exists in *S. cerevisiae* (and other microorganisms). Thus, furfural can be oxidized to furoic acid to some extent in aerobic cultures (Taherzadeh et al. 1999a), and it can be reduced to furfuryl alcohol in anaerobic cultures (Palmqvist et al. 1999a); likewise, 5-hydroxymethylfurfural is reduced to 5-hydroxymethylfurfuryl alcohol under anaerobic conditions (Taherzadeh et al. 2000). Aromatic compounds such as vanillin, hydroxybenzaldehyde, and syringaldehyde are also reduced by the microorganism (Delgenes et al. 1996; Larsson 2000; Larsson et al. 2000).

In confronting the adverse effect of inhibitory compounds found in lignocellulose hydrolysates, preventive aspects include: (1) reducing the formation of inhibitors by utilizing less severe conditions; for instance, addition of SO<sub>2</sub> to steam treatments will minimize production of inhibitors (Stenberg et al. 1998b; Tenborg et al. 1998); (2) chemical detoxification, i.e., by Ca(OH)<sub>2</sub>—"overliming" (Leonard and Hajny 1945; Martinez et al. 2000); (3) appropriate fermentation conditions, for instance, operating in batch mode (Taherzadeh 1999; Taherzadeh et al. 1999b); (4) enzymatic detoxification (Palmqvist et al. 1997; Jönsson et al. 1998); for example the utilization of the phenol-oxidase laccase was identified as a very effi-

cient detoxification method; Larsson et al. 1999b); (5) utilization of inhibitor-tolerant strains: two main strategies, (1) gradual adaptation and (2) genetic engineering, have been used to develop inhibitor tolerance traits. In the first case, the adaptation of *S. cerevisiae* to furfural has been reported in batch (Banerjee et al. 1981a), fed-batch (Villa 1992), and continuous cultures (Chung and Lee 1984). Adaptation was attributed to the synthesis of new enzymes or coenzymes for the reduction of furfural (Boyer et al. 1992), as demonstrated earlier (after 48 h, the levels of ADH increased by 78% when 2 g furfural/l were added to the cultures; Banerjee et al. 1981a). Analogously, a variant of *Z. mobilis*, able to grow on hardwood hydrolysate, was isolated from two sequential continuous cultures that lasted for a total of 149 days. The fraction of hydrolysate in the feed medium for the culture was increased from 10 to 50% (v/v). The isolated strain was capable of tolerating 7.5 g acetate/l in 50% (v/v) hydrolysate. In fermentations of a synthetic hydrolysate containing 4–10 g acetate/l, the ethanol yields reached 94–96% of the theoretical, and productivity was higher than in the unadapted strain (Lawford et al. 1999). The adapted *Z. mobilis* strain was further tested in simultaneous saccharification and fermentation, in which glucose and xylose were converted to ethanol. From dilute-acid-pretreated yellow poplar hardwood, more than 3% (w/v) ethanol was produced and 54% of all sugars (total sugars) were converted in 7 days (McMillan et al. 1999). The great potential of using genetic engineering to obtain inhibitor-resistant *S. cerevisiae* was recently demonstrated by Larsson (2000). Two strategies, the expression of a laccase from *Trametes versicolor* and the overexpression of a phenylacrylic acid decarboxylase, were approached. In the first case, the laccase-expressing strain was able to reduce the aromatic compound coniferyl aldehyde and when grown in diluted acid hydrolysate of spruce, this strain achieved a yield of 0.44 (g ethanol/g fermentable sugars), whereas in the reference strain without laccase the yield was null. In the second case, the overexpression of phenylacrylic acid decarboxylase resulted in a strain resistant to the aromatic compounds ferulic and cinnamic acids; furthermore, when grown in acid hydrolysate of spruce, it achieved higher rates of glucose and mannose consumption and ethanol production (22, 45, and 29% higher, respectively) than the reference strain without gene overexpression.

Perhaps classical techniques can also be useful in generating an acid-/inhibitor-tolerant strain, as demonstrated in the case of ethanol tolerance.

#### *Tolerance to process hardness*

Unlike laboratory bench-scale, large-scale fermentations are carried out with less stringent process control; thus, mixing can be imperfect and undesirable situations may arise. It is therefore important that the microorganism is able to recover from processing errors (Bothast et al. 1999). Some examples of process hardness are: batch

variations in the content of monomeric sugars, oligosaccharides, and inhibitors in pretreated syrup, and in the levels of equipment corrosion residues (McCoy 1998). In addition, there may also be variations in temperature and pH and the presence of contaminant microorganisms.

In simulating process errors, the relative resistance of *E. coli* KO11 was demonstrated by Moniruzzaman et al. (1998). Even after deliberate contamination with up to 10% soil, addition of antibiotics was not required to maintain the maximum catabolic activity of KO11. Fermentation exposed to extremes of temperature (2 h at 5 °C or 50 °C) or pH (2 h at pH 3 or pH 10) recovered after readjustment to the optimal fermentation condition (35 °C, pH 6), although longer times were required for completion in most cases. Ethanol yields were not altered by exposure to extremes in temperature, but were reduced by exposure to extremes in pH. Re-inoculation with 5% (by volume) from control fermentors reduced the delay in fermentation time that otherwise followed the exposure to pH extremes.

## Desirable traits

### *Capability of utilizing sugars simultaneously*

The ideal situation in an industrial setting is to have a microorganism able to consume all sugars simultaneously during growth in a medium containing a mixture of glucose and other sugars. This could result in shorter fermentation time, consequently improving volumetric productivities. But the growth of most microorganisms is diauxic, i.e., when grown in a mixture of sugars, the utilization of other sugars starts only after glucose depletion. This is known as glucose repression. In *S. cerevisiae*, for instance, glucose repression is the outcome of a complex cascade of events. The presence of glucose (or even fructose) is sensed by the cell and triggers an intracellular regulatory cascade that activates Mig1p, a DNA-binding protein (Ronne 1995; Johnston 1999). Mig1p binds to the promoter of alternative sugar-utilization genes, thus blocking the expression of genes encoding metabolism of alternative carbon sources (sucrose, galactose, maltose, etc.).

The benefits of utilizing glucose de-repressed *S. cerevisiae* strains have been demonstrated for sucrose, galactose and maltose, respectively (Olsson et al. 1997; Rønnow et al. 1999; Klein et al. 1996, 1997, 1998, 1999). In the first case, the utilization of sucrose in the presence of glucose by a *Mig1*-disrupted strain was investigated. As a result of the *Mig1* deletion, the specific sucrose hydrolysis rate in the presence of glucose was constant during the fermentation (10 mM/g per h), in contrast to the wild-type in which the sucrose hydrolysis rate was ten-fold lower in the presence of glucose (Olsson et al. 1997).

In the second case, galactose utilization in the presence of glucose was studied. Thus, in a double mutant, *mig1gal80* (*mig1* deletion to avoid glucose repression

and *gal80* deletion to avoid negative regulation of the genes for galactose utilization), galactose consumption during batch cultivation was readily initiated when the glucose concentration was 9.7 g/l, whereas the reference strain showed typical diauxic growth with no galactose consumption in the presence of glucose (Rønnow et al. 1999). Furthermore, the elimination of the three known negative regulators of the *GAL* system – Gal6, Gal80, and Mig1 – resulted in a 41% increased flux in the galactose utilization pathway compared with the wild-type strain, and although biomass formation in the *gal* mutants was not favored, the ethanol production rate increased linearly with the glycolytic flux as a result of the excessive respiro-fermentative metabolism (Ostergaard et al. 2000).

In the third case, the simultaneous utilization of maltose and glucose was investigated. The work done for this purpose serves to illustrate the challenges that metabolic engineering confronts when attempting the improvement of strain characteristics. In recombinant strains with an "industrial background," the disruption in *MIG1* did not alleviate glucose repression, in contrast to laboratory strains (Klein et al. 1996, 1997, 1999). This undesirable and unexpected result emphasized that: (1) the cell is a complex network of regulatory mechanisms, just partially elucidated, which makes it difficult to predict the consequences of the genetic changes introduced; (2) efficient strategies for laboratory strains might not be adequate for industrial strains, since genetic background and genetic characteristics may be different; (3) for industrial purposes, it is important that the introduction of a desirable trait does not adversely affect characteristics such as high specific growth rate and low by-product formation (Olsson and Nielsen 2000).

### *Cellulose/hemicellulose degradation by the microorganism*

The high cost of enzymes needed for the hydrolysis of lignocellulose, is directing research towards the production of low cost lignocellulolytic enzymes and the construction of efficient lignocellulolytic microorganisms. There have been some reports on enabling *S. cerevisiae* to utilize cellulose and hemicellulose. An early work reported that the expression and secretion of  $\beta$ -glucanase (the enzyme that hydrolyzes cellobiose and short oligosaccharides) from *Trichoderma reesei* facilitated the utilization of cellulose and, additionally, improved filterability of the spent medium (Penttilä et al. 1988). More recently, the insertion of genes coding for enzymes that are secreted in the active form has been described. Thus, genes for endo/exo-glucanase and  $\beta$ -glucosidase were chromosomally integrated, generating a strain, *S. cerevisiae* L2612 $\delta$ GC, able to grow and produce ethanol in cellulose-containing media (Cho et al. 1999). There have also been efforts to clone the gene of an effective cellobiase in *Z. mobilis* in order to have a versatile recombinant organism able to ferment glucose and cellulose oli-

gosaccharides (Su et al. 1989). However, the most successful examples up to date involve two bacterial hosts: *Klebsiella oxytoca*, a  $\beta$ -glucosidase producer naturally abundant in paper-mill waste streams (Davis et al. 1992), and *E. coli* KO11 (Moniruzzaman et al. 1997b). *K. oxytoca* naturally transports and utilizes cellobiose and celotriose, the soluble intermediate from cellulose hydrolysis (Ingram et al. 1999).

In *K. oxytoca*, insertion of a plasmid containing the PET operon (see above) and further integration into the chromosome resulted in a *K. oxytoca* strain P2, able to produce ethanol from a variety of monomeric sugars and disaccharides (sucrose, cellobiose, xylobiose), trisaccharides (raffinose, celotriose, xylotriase), and tetrasaccharides (stachiose). Thus, in a medium containing cellulose and two types of commercial cellulases, endoglucanase and exoglucanase, *K. oxytoca* P2 produced ethanol with yields that exceeded 70% of the theoretical (Doran and Ingram 1993). This microorganism also efficiently converted mixed office waste paper (80% cellulose, 10% hemicellulose) into ethanol (Brooks and Ingram 1995). *K. oxytoca* P2 cellulolytic capabilities were further enhanced by chromosomal integration of the *celZ* gene, encoding for endoglucanase in *Clostridium thermocellum*. As heterologous endoglucanase accumulated intracellularly, the subsequent expression of *out* genes (encoding proteins for secretion in *Erwinia chrysanthemii*, a bacterium that transports and utilizes cellobiose and celotriose; Beall and Ingram 1993) in *K. oxytoca* resulted in secretion of more than half of the endoglucanase to the surrounding medium (Ingram et al. 1999; Zhou and Ingram 1999).

In *E. coli* KO11, the approach was to improve its capabilities by insertion of the *casAB* genes (encoding proteins for the transport and utilization of cellobiose and celotriose in *K. oxytoca*). *E. coli* KO11 transformants expressed low levels of the enzymes, but fortunately, spontaneous mutants expressing more than 15-fold higher specific activities were isolated. Three isolated mutants rapidly fermented cellobiose to ethanol, with a yield higher than 90% of the theoretical (Moniruzzaman et al. 1997b).

#### Other desirable traits

There are some traits listed in Table 1 that are desirable in a microorganism, but to which metabolic engineering can make few (if any) contributions, e.g., the GRAS status and the ability to be recycled. Wild-type *S. cerevisiae* and *Z. mobilis* are GRAS organisms; thus, after fermentation, the biomass can be dried and used elsewhere, i.e. as animal feed, without regulatory restrictions. The enteric bacterium *E. coli* lacks GRAS status (Picataggio and Zhang 1996), although PCR investigations have confirmed the absence of all genes known to be associated with pathogenicity in *E. coli* B, the parental strain of KO11 and LY01 (Kuhnert et al. 1997).

Another desirable trait in a microorganism is its reuse in several fermentation cycles, as is the practice with

*S. cerevisiae* in crop-based ethanol processes. It has been estimated that cell recycling in *E. coli* processes could result in up to 28% savings (von Sivers et al. 1994). It remains unclear which processes will enable the microorganism to endure several cycles of fermenting hydrolysates without losing efficiency.

There are, however, opportunities for further tailoring of microorganisms with advantageous traits and to which metabolic engineering and/or classical techniques can contribute:

**Minimal nutrients requirement** Unlike crop-based raw materials, lignocellulose substrates are relatively poor sources of nutrients (Ingram et al. 1998). Nutrient supplementation is therefore mandatory. For example, in KO11 fermentations, nutrients such as corn steep liquor, crude yeast autolysate plus vitamins and minerals, and crude soy hydrolysate plus vitamins and minerals and in *Z. mobilis* corn steep liquor plus diammonium phosphate have been tested (Ingram et al. 1998; Lawford et al. 2000). Although less expensive than peptone or yeast extract, the use of some of these nutrients may still not be satisfactory given the low value of ethanol, that demands minimal, or null nutrient supplementation. As an example, the cost of corn steep liquor supplementation has been estimated as US 0.05/ liter of ethanol (Ingram et al. 1998).

**Growth at low pH** A fermentation conducted at a pH lower than 5.0 has low risk of contamination, and a microorganism fermenting in very acidic conditions would be advantageous.

**Growth at high temperature** Ethanol production is an exergonic process, and during industrial fermentation the reactors must be cooled down to avoid the adverse effects of high temperature on the microorganism. Thus, there are indications that *Z. mobilis* would not need cooling. Interestingly, it has been suggested that the tolerance of a strain to one type of stress, for example temperature, can result in increased osmotolerance and ethanol tolerance (Laluce 1993; Piper 1995); indeed, a common stress-inducible regulator has been identified (Piper et al. 1997). Stress cross-tolerance has been reviewed recently (Jeffries and Jin 2000).

#### Processes for bioethanol production

In the production of bioethanol, several steps are interrelated: feedstock collection, transportation to the manufacturing center, preparation of the raw material, hydrolysis, fermentation, steam generation, product concentration (distillation), and waste disposal (Olsson and Hahn-Hägerdal 1996). These operations need to be fully optimized for an efficient ethanol plant. In Fig. 4, the current processes for crop-based ethanol production and a proposed model for lignocellulose-based ethanol production are compared.

Processes for transformation of crop sugars to ethanol are relatively simple, employing *S. cerevisiae* as the microorganism, and they have been extensively described

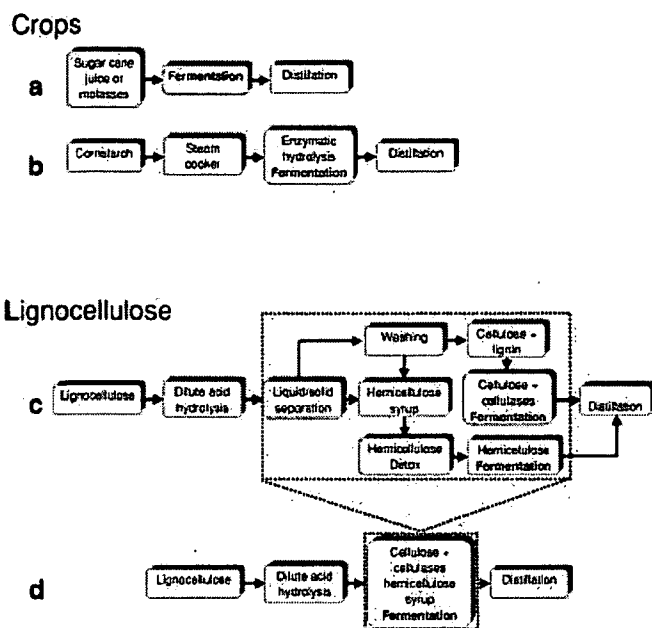


Fig. 4a–d Simplified flowchart for the conversion of biomass to ethanol. Crops: a Sugar cane, b corn. Lignocellulose: c Current model technology, d potential simplified technology. (Adapted from Ingram et al. 1999)

(Kosaric 1996; Wheals et al. 1999). In sugar-cane processing, the microorganism can use the sucrose present in sugar cane juice or molasses (cooked juice) without external hydrolysis. After fermentation, the microorganism can be recycled and the excess of cell biomass dried and used as animal feed. Stillage or vinasse, the distillation byproduct, can, if properly handled, be used as a fertilizer. In corn starch processes, the polymer present in corn and cereals needs hydrolysis before fermentation. The starch is thus gelatinized by cooking at low and high temperature and further hydrolyzed enzymatically, which generates glucose monomers, to be fermented by the microorganism. An outline of crop-based processes is presented in Fig. 4.

For lignocellulose transformation, the degree of complexity is higher (Galbe and Zacchi 1993; Galbe et al. 1997; McMillan 1997) and may require additional steps such as pretreatment, detoxification, and enzyme production (Bothast et al. 1999). Due to their special features, lignocellulose pretreatment, fermentation and waste treatment will be briefly described.

Lynd (1996) has summarized the prerequisites for an ideal lignocellulose pretreatment, it should: (1) produce reactive fibers; (2) yield pentoses in non-degraded form; (3) not lead to the release of compounds that significantly inhibit the fermentation; (4) require little or no size reduction; (5) can work in reactors of reasonable size and moderate cost; (6) produce no solid residues; (7) have a high degree of simplicity, and (8) be effective at low moisture contents. A number of pretreatment options are available: acid pretreatment (at low temperature and concentrated acid, or high temperature and diluted acid),

alkaline pretreatment, steam explosion, AFEX, wet oxidation, organic solvent pretreatment, and hot water (as summarized by Lynd 1996). For example acid pretreatment has the advantage of both releasing sugar monomers from hemicellulose and exposing cellulose fibers for further enzymatic action. Its disadvantage is the generation of inhibitory compounds, so an additional detoxification step might be necessary. Several methods for detoxification are available, as mentioned in a previous section. Although far from being the ideal, acid hydrolysis pretreatment is still the choice in several model processes. Since in industrial scale, water is normally recirculated (Galbe and Zacchi 1993; Palmqvist et al. 1996; Galbe et al. 1997; Larsson et al. 1997), there is a concern that, after each cycle of water utilization, inhibitor levels will build up, which will adversely affect the hydrolytic and fermentation steps by inhibiting cellulases and the microorganism, respectively. In this regard, the development of an inhibitor-tolerant strain may be advantageous, due to the cost of detoxification. In fact, for ethanol production from acid-hydrolyzed willow employing ethanologenic *E. coli* KO11 as the microorganism, it has been estimated that detoxification comprises a significant fraction of the total ethanol production cost (von Sivers et al. 1994).

A fermentation step involves the conversion of sugars from hemicellulose and cellulose. The metabolically engineered microorganism is used in the conversion of hexoses and pentoses of hemicellulose (released by pretreatment) to ethanol. For the hydrolysis of the cellulose component, an enzymatic treatment is preferred. There are a few options when conducting the hydrolysis and fermentation steps: (a) separate hydrolysis and fermentation (SHF), (b) simultaneous saccharification and fermentation (SSF), which uses cellulases from external sources, or (c) simultaneous saccharification and fermentation, in which the microorganism also produces cellulases, in a process often referred to as direct microbial conversion (DMC). These options present advantages and disadvantages. In the first alternative, SHF, each operation can be conducted at optimal conditions of pH and temperature, but the accumulation of the end product of hydrolysis, glucose, inhibits the activity of the cellulases. In the second alternative, SSF, glucose, released by cellulases, is promptly transformed by the microorganism into ethanol. This process has an enhanced rate of hydrolysis, needs lower enzyme loading, results in higher ethanol yields, and reduces the risk of contamination. A compromise is necessary, however, regarding operational temperature. (In processes employing enzymatic hydrolysis, the hydrolytic step lasts longer than the fermentation, so when simultaneous saccharification and fermentation, are used, the overall time is considerably shorter; for this type of process, the evaluation should take this into account). An adaptation of the SSF process has been patented and is known as the Gulf SSF process (Gauss et al. 1976). In the third alternative, DMC, anaerobic *Clostridia* (Ng et al. 1977; Wiegel et al. 1979; Zeikus 1980; Ng et al. 1981, 1982; Ahning et al. 1996;



Lynd 1996) grown at high temperature produce cellulosytic enzymes that hydrolyze the substrate, and the generated sugars are immediately converted to ethanol. The disadvantages are, however, low ethanol yields, caused by byproduct formation (acetate, lactate), low tolerance of the microorganism to ethanol (3.5% w/v), and limited growth in hydrolysate syrups.

The disposal of waste is a relevant issue, given that the driving force for fuel ethanol production and utilization is a cleaner environment (Finley 1981; Szczodrak and Fiedurek 1996). Lignocellulose processes are expected to generate large amounts of waste, including: (1) chemicals that need either recovery for reuse or disposal; (2) cellular biomass after fermentation (GRAS status is important, as discussed previously); (3) waste water generated during the process; (4) vinasse, the distillation byproduct; each liter of ethanol, generates 10–15 l of stillage, as byproduct (Maiorella et al. 1983). Stillage has a very high biochemical oxygen demand (BOD), at 15,000–20,000 ppm. A 100-million-l/year ethanol plant would therefore have a pollution load similar to that of a city of 1.4 million people (Tibellius and Trenholm 1996).

Current proposed processes to produce ethanol from lignocellulose are variations of the Gulf SSF (Ingram et al. 1997; McMillan 1997). A process based on *E. coli* as microorganism is illustrated in Fig. 4C (Ingram et al. 1999). After acid hydrolysis, a solid/liquid separation step follows, and, after washing, the stream (hemicellulose syrup) is CaOH-treated. The resulting detoxified syrup can then be fermented by *E. coli*. Lignin is removed from the solid fraction and can be used as fuel in the plant, whereas cellulose is hydrolyzed by commercial cellulases to generate glucose fermented by *S. cerevisiae*. Alternatively, due to enzyme costs, this step can utilize *K. oxytoca* P2, which requires decreased amount of cellulases. Early economic analysis have predicted that the cost of ethanol production by *E. coli* KO11 utilizing pentose-rich hydrolysate would be US\$0.48/l (von Sivers et al. 1994).

A *Z. mobilis*-based process for the utilization of lignocellulose from hardwoods, primarily poplar species, is being optimized at the National Renewable Energy Laboratory (NREL, USA). The production of cellulases *in situ* is planned to facilitate the simultaneous saccharification and fermentation process. Ideally, these efforts will take the lignocellulose process a step further, closer to the simplified flowchart shown in Fig. 4D (McMillan 1997; OFD 1999). The estimated cost for ethanol in this process is US\$0.36/l. Some optimistic forecasts count on advanced biotechnological tools, i.e., metabolic engineering, to improve yields and performance of the conversion process and to reduce costs of ethanol from lignocellulose by US\$0.11/l in the next 10 years (Wooley et al. 1999).

The USA is leading world efforts to develop a competitive process for lignocellulose ethanol, acting through several governmental agencies. Recently the Office for Fuel Development (OFD) proposed ideas to reduce ethanol costs: (a) the development of a counter-

current reactor for the pretreatment of biomass; (b) methods for processing lignin residues for new, higher value products; (c) the integration of all unit operations; (d) the evaluation and optimization of process configurations. As an example, current research funded by that agency involves: (a) advanced pretreatments to increase sugar yields and reduce sugar degradation; (b) improved cellulase and hemicellulase enzymes; (c) consolidated bioprocessing of hydrolysis and fermentation; (d) product diversification, including coproduction of non-fuel products (e.g., organic chemicals and bio-based material) with bioethanol.

## Future prospects

The utilization of fuel ethanol for transportation has the potential to contribute to a cleaner environment. It is expected that the bioethanol industry will benefit from the efficient utilization of lignocellulose. Technical achievements in this emerging sector, particularly the satisfactory performance of metabolically engineered microorganisms in pilot scale, have favored optimistic forecasts.

Meanwhile, metabolic engineering (in conjunction with classical techniques such as random mutagenesis) is addressing the further enhancement of microorganism capabilities by adding/modifying traits such as tolerance to ethanol and inhibitors, hydrolysis of cellulose/hemicellulose, thermotolerance, reduced need nutrient supplementation, and improvement of sugars transport.

However, keeping a realistic perspective is important. The improvement achieved in the fermentation step with the help of metabolic engineering is just one of the aspects of an integrated process. Hence, several pieces still remain to be properly assembled (and optimized) before an efficient industrial configuration is acquired. It is therefore anticipated that once in operation, the current model technologies will need several cycles of improvement/analysis, before optimization and competitiveness are achieved.

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## Ethanol fermentation from biomass resources: current state and prospects

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**Abstract** In recent years, growing attention has been devoted to the conversion of biomass into fuel ethanol, considered the cleanest liquid fuel alternative to fossil fuels. Significant advances have been made towards the technology of ethanol fermentation. This review provides practical examples and gives a broad overview of the current status of ethanol fermentation including biomass resources, microorganisms, and technology. Also, the promising prospects of ethanol fermentation are especially introduced. The prospects included are fermentation technology converting xylose to ethanol, cellulase enzyme utilized in the hydrolysis of lignocellulosic materials, immobilization of the microorganism in large systems, simultaneous saccharification and fermentation, and sugar conversion into ethanol.

### Introduction

With the inevitable depletion of the world's energy supply, there has been an increasing worldwide interest in alternative sources of energy (Aristidou and Penttila 2000; Jeffries and Jin 2000; John 2004; Kerr 1998; Wheals et al. 1999; Zaldivar et al. 2001). It is now understood that it is important to use biomass energy as a means of providing modern energy to the billions who lack it. It would complement solar, wind, and other intermittent energy sources in the renewable energy mix of the future. One of the most immediate and important applications of biomass energy systems could be in the fermentation of ethanol from biomass.

Biomass is seen as an interesting energy source for several reasons. The main reason is that bioenergy can con-

tribute to sustainable development (Van den Broek 2000; Monique et al. 2003). Resources are often locally available, and conversion into secondary energy carriers is feasible without high capital investments. Moreover, biomass energy can play an important role in reducing greenhouse gas emissions; since CO<sub>2</sub> that arises from biomass wastes would originally have been absorbed from the air, the use of biomass for energy offsets fossil fuel greenhouse gas emissions (Lynd 1996). Furthermore, since energy plantations may also create new employment opportunities in rural areas, it also contributes to the social aspect of sustainability. In addition, application of agro-industrial residues in bioprocesses not only provides alternative substrates but also helps solve their disposal problem. With the advent of biotechnological innovations, mainly in the area of enzyme and fermentation technology, many new avenues have opened for their utilization.

Nearly all fuel ethanol is produced by fermentation of corn glucose in the US or sucrose in Brazil (MacDonald et al. 2001; Rosillo-Calle and Cortez 1998), but any country with a significant agronomic-based economy can use current technology for fuel ethanol fermentation. This is possible because, during the last two decades, technology for ethanol production from nonfood-plant sources has been developed to the point at which large-scale production will be a reality in the next few years. Therefore, agronomic residues such as corn stover (corn cobs and stalks), sugarcane waste, wheat or rice straw, forestry, and paper mill discards, the paper portion of municipal waste and dedicated energy crops—collectively termed “biomass”—can be converted into fuel ethanol. In this field, although bioethanol production has been greatly improved by new technologies, there are still challenges that need further investigations. A further understanding of the ethanol fermentation needs to be reached.

This review will focus on the current status of ethanol fermentation including biomass resources, microorganisms, technology, the practical examples, and especially the promising prospects of ethanol fermentation.

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## Biomass resources

There are various forms of biomass resources in the world, which can be grouped into four categories. Wood residues are by far the largest current source of biomass for energy production. It comes from the wood product industry which includes paper mills, sawmills, and furniture manufacturing. Municipal solid waste is the next largest, followed by agriculture residues and dedicated energy crops. Among these biomass resources including short-rotation woody crops and herbaceous crops, primarily tall grasses, dedicated energy crops seem to be the largest, most promising, future resource of biomass. This is because of the ability to obtain numerous harvests from a single planting, which significantly reduces average annual costs for establishing and managing energy crops, particularly in comparison to conventional crops (Monique et al. 2003).

Fermentation processes from any material that contains sugar could derive ethanol. The varied raw materials used in the manufacture of ethanol via fermentation are conveniently classified into three main types of raw materials: sugars, starches, and cellulose materials. Sugars (from sugarcane, sugar beets, molasses, and fruits) can be converted into ethanol directly. Starches (from corn, cassava, potatoes, and root crops) must first be hydrolyzed to fermentable sugars by the action of enzymes from malt or molds. Cellulose (from wood, agricultural residues, waste sulfite liquor from pulp, and paper mills) must likewise be converted into sugars, generally by the action of mineral acids. Once simple sugars are formed, enzymes from microorganisms can readily ferment them to ethanol.

The most widely used sugar for ethanol fermentation is molasses which contains about 50 wt% of sugar and about 50 wt% of organic and inorganic compounds, including water. It is a thick, dark-colored syrup produced during refinement of sugar. Since molasses contains microorganisms which can disturb the fermentation, the molasses is taken first to the sterilizer and then to the fermentor. Then it is diluted with water to the mass fraction of  $10 \pm 18\%$  to reduce its viscosity in the pipeline. In addition, a very high concentration of sugar can give too much ethanol and results in a prolonged fermentation time and an incomplete sugar conversion. After the pH of the mash is adjusted to about 4–5 with mineral acid, it is inoculated with yeast or bacteria, and the fermentation is carried out nonaseptically at 20–32°C for about 1–3 days.

Most agricultural biomass containing starch can be used as a potential substrate for the ethanol fermentation by microbial processes. These substrates include corn (maize), wheat, oats, rice, potato, and cassava. On a dry basis, corn, wheat, sorghums (milo), and other grains contain around 60–75% (wt/wt) of starch, hydrolyzable to hexose with a significant weight increase (stoichiometrically the starch to hexose ratio is 9:10), and these offer a good resource in many fermentation processes (Jackman 1987).

Fermentation of starch is somewhat more complex than fermentation of sugars because starch must first be converted into sugar and then into ethanol. Starch is first hydrolyzed by adding  $\alpha$ -amylase to avoid gelatinization,

then cooked at high temperature (140–180°C). Next, the liquefied starch is hydrolyzed to glucose with glucoamylase. The resulting dextrose is fermented to ethanol with the aid of microorganisms producing  $\text{CO}_2$  as a coproduct. During the process currently employed for industrial-scale ethanol fermentation from starchy materials, high-temperature cooking (140–180°C) is very effective for fermentation of starchy materials because it raises starch saccharification efficiency and achieves high levels of ethanol production under complete sterilization of harmful microorganisms. However, production costs are high due to the high energy consumption in the cooking process and the addition of large amounts of amylolytic enzymes. So processes to reduce the high production costs are required. To resolve these difficulties, noncooking and low-temperature cooking fermentation systems have been developed (Matsumoto et al. 1985).

Industrial ethanol production has been reported using various starchy materials such as corn, wheat, starch and potatoes, cassava root (Lindeman and Rocchiccioli 1979), corn stover (Kadam and McMillan 2003; Wilke et al. 1981), and starch (Maisch et al. 1979). Among many starchy materials, cassava starch is an inexpensive fermentable source. It is a tropical root crop produced in more than 80 countries (Sasson 1990). About 20% of the cassava starch was incorporated into animal feed. A similar amount was converted into starch for industrial use and another portion used for human food in some developing countries. The rest was lost since cassava is perishable after harvest. Harnessing the lost portion in addition to gains from new high-yielding varieties with outputs of 100 tons per hectare could provide the fermentation industry with an abundance of raw material (Anthony et al. 1996). Fresh cassava has a very high starch content, up to 30%. The content of sucrose is about 4%. Dried cassava has 80% fermentable substrate.

However, cassava waste processing is difficult because it is high in toxic materials. The potential toxicity of cassava is due to the presence of cyanogenic glycosides, linamarin, and lotaustralin, which on hydrolysis yield hydrogen cyanide on its peel. Traditional methods of cooking like boiling and decanting remove cyanoglycosides to a certain extent, but even then a certain amount of residual toxicity remains in it (Westley 1980). Moreover, since starch particles in cassava are bigger and there are some branched structures, more glucoamylase has to be added into the reactor. Furthermore, the nitrogen content of the cassava is very low, so during the fermentation, nutrient has to be added into the reactor to maintain the normal growth of the microorganisms.

Among the three main types of raw materials, cellulose materials represent the most abundant global source of biomass and have been largely unutilized. The global production of plant biomass, of which over 90% is lignocellulose, amounts to about  $200 \times 10^9$  tons per year, where about  $8\text{--}20 \times 10^9$  tons of the primary biomass remains potentially accessible. However, the effective utilization of the lignocellulosic feedstock is not always practical because of its seasonal availability, scattered stations, and the high costs of transportation and storage of such large amounts of organic

material (Polman 1994). Recently, the enzymatic hydrolysis of biomass cellulose is considered to be the most promising technology available (Ogier et al. 1999; Yu and Zhang 2004). However, despite the work done, the industrial scale-up of this process appears to be still hindered by technological issues or by the lack of a biomass refinery approach in which ethanol is one of several products. In fact, because raw material cost comprises more than 20% of the production cost (Brown et al. 2001; Kaylen et al. 2000; Zhuang et al. 2001), the optimization of the cellulose conversion should be accomplished by correct management and utilization of all process streams. A consequence of this situation is that even limited government intervention is still crucial to maintaining ongoing research.

Furthermore, lignocellulose is a more complex substrate than starch. It is composed of a mixture of carbohydrate polymers (cellulose and hemicellulose) and lignin. The carbohydrate polymers are tightly bound to lignin mainly by hydrogen bonds but also by some covalent bonds. The biological process for converting the lignocellulose to fuel ethanol requires the following: delignification to liberate cellulose and hemicellulose from their complex with lignin, depolymerization of the carbohydrate polymers to produce free sugars, and fermentation of mixed hexose and pentose sugars to produce ethanol. Among the key processes described above, the delignification of lignocellulosic raw materials is the rate-limiting and most difficult task to be solved. Another problem is that the aqueous acid used to hydrolyze the cellulose in wood to glucose and other simple sugars destroys much of the sugars in the process. Extensive research has been carried out in this field for decades (Yu and Zhang 2004), and the first demonstration plant using lignocellulosic feedstocks has been in operation in Canada since April 2004 (Tampier et al. 2004). It is expected that the cost of lignocellulosic ethanol can undercut that of starch-based ethanol because low-value agricultural residues can be used.

### General process

Besides the initial removal of large and unsuitable items, key components of an integrated residual waste treatment system based on ethanol fermentation include recyclable materials recovery and removal of contaminants via mechanical preprocessing, initial hydrolysis process (conversion to simpler compounds), fermentation of organics, postfermentation purification of ethanol (by distillation or filtration), gasification of solid residuals to provide process heat, and treatment and disposal of wastewater.

Nearly all of the ethanol fermentation technologies use an initial tipping floor removal of large or unsuitable materials, followed by mechanical preprocessing to remove recyclables and contaminants, and shredding of the material. Then the material is processed through vessels using various systems for the purpose of hydrolysis (breaking down to simpler compounds) of the material. Depending on the technology, this may include high temperature, acid treatment, and/or high pressure. Following the initial hydrolysis

phase, the slurried material is then fermented to produce alcohol, which is then purified through distillation and/or filtration to produce the desired fuel-grade quality ethanol.

When cellulose was used as the raw material, the cellulase responsible for enzymatic hydrolysis of pretreated cellulosic biomass is strongly inhibited by hydrolysis products: glucose and short cellulose chains. One way to overcome cellulase inhibition is to ferment the glucose to ethanol as soon as it appears in solution. Simultaneous saccharification and fermentation (SSF) combines enzymatic hydrolysis with ethanol fermentation to keep the concentration of glucose low (as shown in Fig. 1). The accumulation of ethanol in the fermentor does not inhibit cellulase as much as high concentrations of glucose, so SSF is a good strategy for increasing the overall rate of cellulose to ethanol conversion. In comparison to the process where these two stages are sequential, the SSF method enables attainment of higher (up to 40%) yields of ethanol by removing end-product inhibition, as well as by eliminating the need for separate reactors for saccharification and fermentation (Bollók et al. 2000; Hari et al. 2001; Stenberg et al. 2000). Other advantages of this approach are a shorter fermentation time and a reduced risk of contamination with external microflora, due to the high temperature of the process, the presence of ethanol in the reaction medium, and the anaerobic conditions (Emert and Katzen 1980; Wyman 1994).

In spite of the obvious advantages presented by the SSF, it has some drawbacks. These lie mainly in different temperature optima for hydrolysis (45–50°) and fermentation (28–35°) (Ballesteros et al. 2004; Jeffries and Jin 2000; Jeffries and Shi 1999). Besides, ethanol itself and some toxic substances arising from pretreatment of the lignocellulose inhibit the action of fermenting microorganisms, as well as the cellulase activity (Targonski and Achremowicz 1986; Yu and Zhang 2004). Achieving microorganism-enzyme compatibility becomes a major issue in the SSF, since some compounds (e.g., proteolytic enzymes) that are released on cell lysis or are secreted by a particular strain can degrade the cellulases, alternately, components in the enzyme preparation, and reduce microbial viability leading to cell lysis. On the whole, several process parameters must be optimized: substrate concentration, enzyme to substrate ratio, dosage of the active components ( $\alpha$ -glucosidase to glucanase ratio) in the enzymatic mixture, and yeast concentration.

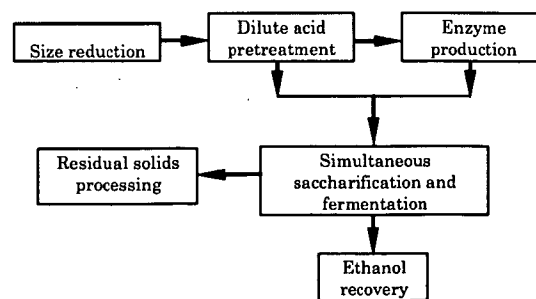


Fig. 1 Schematic diagram of the conversion of biomass feedstock to ethanol fuel



## Microorganisms related to ethanol fermentation

Ethanol fermentation is a biological process in which organic material is converted by microorganisms to simpler compounds, such as sugars. These fermentable compounds are then fermented by microorganisms to produce ethanol and CO<sub>2</sub>. During the whole process of ethanol fermentation, there are mainly two parts for microorganisms. One is for the microorganisms which convert fermentable substrates into ethanol, and the other is to produce the enzyme to catalyze chemical reactions that hydrolyze the complicate substrates into simpler compounds.

### Microorganisms producing ethanol

Several reports and reviews have been published on production of ethanol fermentation by microorganisms, and several bacteria, yeasts, and fungi have been reportedly used for the production of ethanol. Those microbes that are capable of yielding ethanol as the major product are shown in Tables 1 and 2.

As shown in Tables 1 and 2, there are some microorganisms which can accumulate high concentrations of ethanol. Historically, the most commonly used microbe has been yeast, among the yeasts, *Saccharomyces cerevisiae*, which can produce ethanol to give concentration as high as 18% of the fermentation broth, is the preferred one for most ethanol fermentation. This yeast can grow both on simple sugars, such as glucose, and on the disaccharide sucrose. *Saccharomyces* is also generally recognized as safe (GRAS) as a food additive for human consumption and is therefore ideal for producing alcoholic beverages and for leavening bread.

As with many microorganisms, *S. cerevisiae* metabolizes glucose by the Embden–Meyerhof (EM) pathway. Beside this, the Entner–Doudoroff (ED) pathway is an additional means of glucose consumption in many bacteria, such as *Zymomonas*. The high ethanol yield and productivity observed for *Zymomonas* are a consequence of its unique physiology. *Zymomonas* is the only microorganism that metabolizes glucose anaerobically using the ED pathway as opposed to the EM or glycolytic pathway (Matthew et al. 2005). The ED pathway yields only half as much ATP per mole of glucose as the EM pathway. As a consequence, *Zymomonas* produces less biomass than yeast, and more carbon is funneled to fermentation products. Also, as a consequence of the low ATP yield, *Zymomonas* maintains a high glucose flux through the ED pathway. All the enzymes involved in fermentation are expressed constitutively, and fermentation enzymes comprise as much as 50% of the cells' total protein (Sprenger 1996).

*Zymomonas mobilis* is an unusual Gram-negative microorganism that has several appealing properties as a biocatalyst for ethanol production. The microorganism has a homoethanol fermentation pathway and tolerates up to 120 g/l ethanol. It has a higher ethanol yield (5–10% more ethanol per fermented glucose) and has a much higher specific ethanol productivity (2.5×) than *Saccharomyces*

sp. (Sprenger 1996). Furthermore, *Z. mobilis* is GRAS and has simple nutritional needs. It is so well suited for ethanol production that in the 1970s and 1980s, some researchers advocated it as superior to *S. cerevisiae*. Despite its advantages as an ethanologen, *Z. mobilis* is not well suited for all of the biomass resources conversion because it ferments only glucose, fructose, and sucrose. Moreover, for *Z. mobilis* on synthetic media containing either glucose, fructose or sucrose, the specific rates of sugar uptake and ethanol production are at a maximum when utilizing the glucose medium. In addition, *S. cerevisiae* is still preferred by the industry because of the yeast hardiness.

Engineering *Escherichia coli* is another valuable bacterial resource for ethanol production. The construction of *E. coli* strains to selectively produce ethanol (Millichip and Doelle 1989) was one of the first successful applications of metabolic engineering. *E. coli* has several advantages as a biocatalyst for ethanol production, including the ability to ferment a wide spectrum of sugars, no requirements for complex growth factors, and prior industrial use (e.g., for production of recombinant protein). The major disadvantages associated with using *E. coli* cultures are a narrow and neutral pH growth range (6.0–8.0), less hardy cultures compared to yeast, and public perceptions regarding the danger of *E. coli* strains. The lack of data on the use of residual *E. coli* cell mass as an ingredient in animal feed is also an obstacle to its application.

Cellulose-to-ethanol biotransformation can be conducted by various anaerobic thermophilic bacteria, such as *Clostridium thermocellum* (Ingram et al. 1987), as well as by some filamentous fungi, including *Monilia* sp. (Saddler and Chan 1982), *Neurospora crassa* (Gong et al. 1981), *Neurospora* sp. (Yamauchi et al. 1989), *Zygosaccharomyces rouxii* (Pastore et al. 1994), *Aspergillus* sp. (Sugawara et al. 1994), *Trichoderma viride* (Ito et al. 1990), and *Paecilomyces* sp. (Gervais and Sarrette 1990). However, studies on the fermentation process utilizing these microorganisms have shown this process to be very slow (3–12 days) with a poor yield (0.8–60 g/l of ethanol), which most probably is due to the low resistance of microorganisms to higher concentrations of ethyl alcohol. Another disadvantage of this process (particularly in the case of bacterial fermentation) is the production of various by-products, primarily acetic and lactic acids (Herrero and Gomez 1980; Wu et al. 1986).

### Hydrolysis enzymes and the related microorganisms

In addition to polymeric carbohydrates, raw material for ethanol fermentation contains varying amounts of polyphenolic lignin and other "extractables." These compounds are not directly fermentable by most yeasts, and they must be pretreated to hydrolyze the complicate compounds to simple sugars (Zertuche and Zall 1982). Development of an ideal pretreatment process is difficult, given that "biomass" includes such sources as hardwood and softwood trees, agricultural residues such as corn stover and nonrecyclable paper waste.



**Table 1** Yeast species which produce ethanol as the main fermentation product

Strain-species	Temperature (°C)	pH value	Carbon source and concentration (g/l)	Nitrogen source and concentration (g/l)	Incubation time (h)	Concentration of ethanol produced (g/l)	References
27817- <i>Saccharomyces cerevisiae</i>	30	5.5	Glucose (50–200)	Peptone (2) and ammonium sulfate (4)	18–94	5.1–91.8	Vallet et al. 1996
L-041-S. <i>cerevisiae</i>	30 or 35	–	Sucrose (100)	Urea (1) or ammonium sulfate (1–2)	24	25–50	Leticia et al. 1997
181-S. <i>cerevisiae</i> (aerobic)	27	6.0	Glucose (10)	Peptone (5.0)	40–160	–	Todor and Tsonka 2002
UO-1-S. <i>cerevisiae</i> (aerobic)	30	5.0	Sucrose (20)	Ammonium sulfate (1)	60–96	–	Camacho-Ruiz et al. 2003
V5-S. <i>cerevisiae</i>	24	–	Glucose (250)	–	36	–	Virginie et al. 2001
ATCC 24860-S. <i>cerevisiae</i>	30	4.5	Molasses (1.6–5.0)	Ammonium sulfate (0.72–2.0)	24	5–18.4	Ergun and Mutlu 2000
Bakers' yeast-S. <i>cerevisiae</i>	30	4.5	Sugar (150–300)	–	192	53 (max)	Roukas 1996
Bakers' yeast-S. <i>cerevisiae</i>	28	5.0	Sucrose (220)	Peptone(5) and ammonium dihydrogen phosphate (1.5)	96	96.71	Caylak and Vardar 1996
Fiso-S. <i>cerevisiae</i>	30	5.0	Galactose (20–150)	Peptone, ammonium sulfate and casamino acid (10)	60	4.8–40	da Cruz et al. 2003
A3-S. <i>cerevisiae</i>	30	5.0	Galactose (20–150)	Peptone, ammonium sulfate and casamino acid (10)	60	4.8–36.8	da Cruz et al. 2003
L52-S. <i>cerevisiae</i>	30	5.0	Galactose (20–150)	Peptone, ammonium sulfate and casamino acid (10)	60	2.4–32.0	da Cruz et al. 2003
GCB-K5-S. <i>cerevisiae</i>	30	6.0	Sucrose (30)	Peptone (5)	72	27	Kiran et al. 2003
GCA-II-S. <i>cerevisiae</i>	30	6.0	Sucrose (30)	Peptone (5)	72	42	Kiran et al. 2003
KR <sub>18</sub> -S. <i>cerevisiae</i>	30	6.0	Sucrose (30)	Peptone (5)	72	22.5	Kiran et al. 2003
CMI237-S. <i>cerevisiae</i>	30	4.5	Sugar (160)	Ammonium sulfate (0.5)	30	70 (max)	Navarro et al. 2000
2.399-S. <i>cerevisiae</i>	30	5.5	Glucose (31.6)	Urea (6.4)	30	13.7 (max)	Yu and Zhang 2004
24860-S. <i>cerevisiae</i>	–	–	Glucose (150)	Ammonium dihydrogen phosphate (2.25)	27	48 (max)	Ghasem et al. 2004
27774- <i>Kluyveromyces fragilis</i>	30	5.5	Glucose (20–120)	Peptone (2) and ammonium sulfate (4)	18–94	48.96 (max)	Vallet et al. 1996
30017-K. <i>fragilis</i>	30	5.5	Glucose (20–120)	Peptone (2) and ammonium sulfate (4)	18–94	48.96 (max)	Vallet et al. 1996
30016- <i>Kluyveromyces marxianus</i>	30	5.5	Glucose (100)	Peptone (2) and ammonium sulfate (4)	18–94	44.4 (max)	Vallet et al. 1996
30091- <i>Candida utilis</i>	30	5.5	Glucose (100)	Peptone (2) and ammonium sulfate (4)	18–94	44.4 (max)	Vallet et al. 1996
ATCC-32691 <i>Pachysolen tannophilus</i>	30	4.5	Glucose (0–25) and xylose (0–25)	Peptone (3.6) and ammonium sulfate (3)	100	7.8 (max)	Sanchez et al. 1999

**Table 2** Bacterial species which produce ethanol as the main fermentation product

Mesophilic organisms	Mmol ethanol produced per mmol glucose metabolized	References
<i>Clostridium sporogenes</i>	up to 4.15 <sup>a</sup>	Miyamoto 1997
<i>Clostridium indoli</i> (pathogenic)	1.96 <sup>a</sup>	Miyamoto 1997
<i>Clostridium sphenoides</i>	1.8 <sup>a</sup> (1.8) <sup>b</sup>	Miyamoto 1997
<i>Clostridium sordelli</i> (pathogenic)	1.7	Miyamoto 1997
<i>Zymomonas mobilis</i> (syn. <i>Anaerobica</i> )	1.9	Miyamoto 1997
<i>Zymomonas mobilis</i> subsp. <i>pomaceas</i>	1.7	Miyamoto 1997
<i>Spirochaeta aurantia</i>	1.5 (0.8)	Miyamoto 1997
<i>Spirochaeta stenostrepta</i>	0.84 (1.46)	Miyamoto 1997
<i>Spirochaeta litoralis</i>	1.1 (1.4)	Miyamoto 1997
<i>Erwinia amylovora</i>	1.2	Miyamoto 1997
<i>Escherichia coli</i> KO11	0.7–0.1	Dien et al. 2003; Matthew et al. 2005
<i>Escherichia coli</i> LY01	40–50 g ethanol produced/l	Dien et al. 2003
<i>Leuconostoc mesenteroides</i>	1.1	Miyamoto 1997
<i>Streptococcus lactis</i>	1.0	Miyamoto 1997
<i>Klebsiella oxytoca</i>	0.94–0.98	Matthew et al. 2005
<i>Klebsiella aerogenes</i>	24 g ethanol produced/l	Ingram et al. 1998
<i>Mucor</i> sp. M105	–	Ingram et al. 1998
<i>Fusarium</i> sp. F5	–	Ingram et al. 1998

These diverse feedstocks have caused researchers to test numerous pretreatment processes ranging from hot water and steam explosion treatments, to alkaline and solvent pretreatments, to many useful versions of acid pretreatment (Kaar and Holtzapple 2000; Maiorella 1985; Sun and Cheng 2002). However, they acknowledge that detoxification of acid-hydrolyzed lignin and other “extractables” in the sugar hydrolysate will present additional costs for the total hydrolysis process, costs that could be avoided en-

tirely if a fully enzymatic process (yet to be developed) is implemented instead.

Traditionally, starch was, and still is, hydrolyzed to low molecular weight dextrans and glucose using acid, but enzymes have several advantages. First, the specificity of enzymes allows the production of sugar syrups with well-defined physical and chemical properties. Second, the milder enzymatic hydrolysis results in few side reactions and less “browning.” Indeed, for the production of glucose syrups from starch, enzymic hydrolysis is essential. A summary of starch degrading enzymes is shown in Fig. 2 (Hsu 1996).

There have been several reports about yeasts that could produce extracellular  $\alpha$ -amylase and glucoamylase. These include *Candida tsukubaensis* CBS 6389 (Aktinson and Mavituna 1991), *Filobasisium capsuligenum* (Aktinson and Mavituna 1991), *Lipomyces kononenkoae* (de Mot and Verachtert 1985), *Lipomyces starkeyi* (Spencer-Martins and Van Uden 1979), *Saccharomycopsis bispore* (formerly *Endomycopsis bispore*) (Kelly et al. 1985), *Saccharomycopsis capsularis*, *Saccharomycopsis fibuligera* (Ebertova 1966; Stepanov et al. 1975), *Schwanniomyces alluvius* (Gasperi et al. 1985), *Schwanniomyces castelli* (Simoes-Mendes 1984), and *Trichosporon pullulans* (Silla et al. 1984).

In addition, for the production of cellulolytic enzymes to be used in the hydrolysis, the lignocellulose-degrading fungus *Trichoderma reesei* can be used (Sharma 2000). This fungus is able to metabolize pentose and hexose sugars and also oligomers, and it is insensitive to inhibitors generated from the lignocellulosic material, because these are normally present in its natural environment.

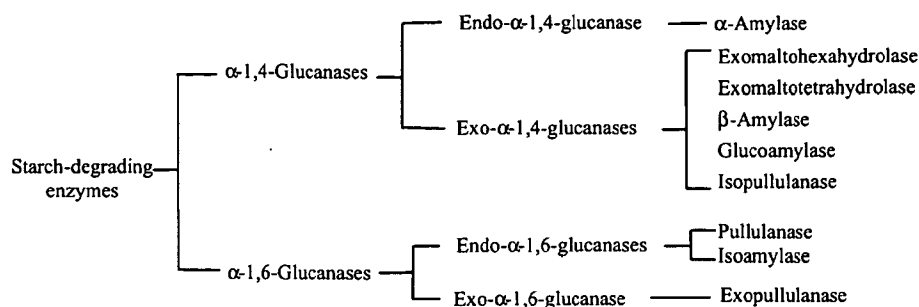
In this field, it was investigated whether the cellulolytic fungus *T. reesei* could degrade inhibitory compounds present in a hemicellulose hydrolysate obtained after steam pretreatment of willow and thereby decrease its inhibitory effect on the ethanolic fermentation by *S. cerevisiae*. It was also investigated whether the inhibitor containing fraction could be used as a carbon source for the production of high-quality cellulolytic enzymes to be used in the hydrolysis.

### Kinetic models

Generally, economic restrictions force industrial processes to work in a very small range of operating conditions. For some batch processes which have long operating times in each cycle and depend strongly on the operating variables, it is very important to define the optimum conditions to achieve sufficient profitability. Kinetic models describing the behavior of microbiological systems can be a highly appreciated tool and can reduce tests to eliminate extreme possibilities.

Various kinetic models have been proposed in the literature for freely suspended cells in either batch or continuous operation (Ramon-Portugal et al. 1997; Reynnders et al. 1996; Tan et al. 1996). Unstructured models give the most fundamental observations concerning microbial metabolic processes and can be considered a good approxi-

**Fig. 2** A summary of starch degrading enzymes



mation when the cell composition is time dependent or when the substrate concentration is high compared to the saturation constant (Sonnleitner et al. 1997). Control models for routine operation of industrial fermentations are often based on simple, unstructured models since the process computer will adjust the model parameters based on the response of the system to disturbances.

When cultured in glucose media, unstructured models have been found effective for describing the exponential phase of the batch fermentation kinetics of cell growth and ethanol production for strains of *Z. mobilis* ZM4 and ATCC 10988 (Moser 1985). These models, incorporated with the bottleneck model approach, provide a base for establishing a structured model that can describe the transient behavior of a batch fermentation. An additional parameter, reflecting the quality of the inoculum, is adjusted to match the model prediction with the corresponding experimental result. In continuous culture, the experimental findings suggest that the specific substrate uptake rate is not linearly dependent upon the specific growth rate,  $\mu$ . A structured two-compartment model was introduced by Jobses et al. (1985) to describe the fermentation of *Z. mobilis*. According to this model, the specific substrate (glucose) uptake rate in steady-state continuous culture is a nonlinear second-order function of  $\mu$ .

Gulnur et al. (1998) investigated the mathematical description concerned with the basic metabolic processes of *S. cerevisiae* in immobilized form. Glucose utilization, ethanol production, and growth pattern of yeast cells immobilized in calcium alginate gel beads were determined in a stirred batch system using four different initial substrate concentrations. Eleven different mathematical models taking into account the possibility of glucose or ethanol inhibition on both yeast cell growth and ethanol production were studied. The batch performance curves predicted by the models were compared with the experimental data, and the results were analyzed in terms of the possible effects of initial condition (Doruker et al. 1995).

During the simulation of batch alcoholic fermentation with the different initial conditions employed 11 different models: the models of Monod, Moser, and Teissier were used to represent inhibition-free substrate limitation kinetics; the models of Andrews and Noack, Aiba and Luong include substrate inhibition effects, whereas the models of Levenspiel, Aiba, Jerusalimsky, Ghose and Tyagi, and

Hinshelwood include product inhibition effects. The models proposed by Monod and Hinshelwood were found to be more appropriate for describing the batch growth and ethanol production of immobilized *S. cerevisiae* at low and high initial glucose concentrations, respectively (Gulnur et al. 1998).

Structured models describing culture kinetics are important in the control of bioreactors, as they provide a mathematical description of the mechanism of the process which are required for optimization and control. The objective of structured modeling is to obtain expressions that quantitatively describe the behavior of the process under consideration. A wide variety of models have been proposed for the kinetics of the process; these range from very simple models (Mori et al. 1970; Namba et al. 1984) to more complex global models (Park and Toda 1990; Park et al. 1990, 1991), which take into account the activating and inhibiting effects of the substrate (glucose and oxygen) and the product (ethanol and acetic acid; Oh et al. 2000). However, none of these studies have put forward a general model sufficiently well developed to permit the design of a good simulator which is capable of performing simulations with batch processes.

Moreover, structured models have been used to predict the influence of operating parameters on cell concentration, substrate utilization rate, and ethanol production rate. These models may lead to the development of better strategies for the optimization of the fermentation process to ensure its economic viability. Although four factors (substrate limitation, substrate inhibition, product inhibition, and cell death) are known to affect ethanol fermentation, none of these models accounts for these kinetic factors simultaneously. Monod's (1950) equation accounts only for substrate limitation. The models of Hinshelwood (1946), Holzberg et al. (1967), Egamberdiev and Jerusalimsky (1968), Nagatani et al. (1968), Ghose and Tyagi (1979), Hoppe and Hansford (1982), and Lee (1988) account only for ethanol inhibition. The models of Aiba et al. (1968), Aiba and Shoda (1969), and Luong (1985) include only substrate limitation and substrate inhibition terms. An appropriate ethanol fermentation model should therefore account for the four kinetic factors.

A developed mathematical model capable of predicting the cell, substrate, and ethanol concentrations during the continuous anaerobic fermentation is necessary. However,

it cannot be expected that any kinetic model will be directly applicable to a real process situation. Therefore, mathematical modeling should start with the simplest type, but it must be reiterated, modified, and extended until it eventually leads to an adequate process kinetic model.

### Pilot plants producing ethanol

Approximately 80% of the ethanol produced in the world is still obtained from fermentations; the remainder comes largely by synthesis from the petroleum product, ethylene. The alcohol produced in the US is primarily used in alcoholic beverages, but this is not always the case elsewhere in the world. Brazil has embarked on a major program to produce ethanol for fuel and thereby diminish petroleum imports. As of 1984, approximately 7.9 million tons of ethanol was produced by fermentation in Brazil, with sucrose from sugarcane as the carbon source. The US is also substantially increasing its fuel alcohol production, originally because of the rapid increase in petroleum costs during the 1970s, and the subsequent need for developing alternative energy sources.

In spite of extensive research on fuel ethanol production from biomass (shown in Table 3), until 1995, not a single plant capable of converting cellulosic feedstock to ethanol, via biological processing on the industrial scale, has been put into operation anywhere in the world, although some pilot scale plants have been commissioned (Szczo drak and Fiedurek 1996).

During World War II, when wartime conditions changed economic conditions and priorities, several ethanol-from-cellulose (EFC) plants were built and operated in various countries to provide an alternative fuel source. These countries include Germany, Russia, China, Korea, Switzerland, and the US among others. Since the end of the war, competition from synthetically produced ethanol has forced many of these plants to close (Badger 2002). Since April 2004, the first demonstration plant using lignocellulosic

feedstocks in Canada has been in operation (Tampier et al. 2004). The target volume of 100 million liters of ethanol, anticipated by 2006, will likely be met or exceeded by 2007. There is also progress on pretreatment of softwood residues and pentose fermentation (Natural Resources Canada's management team 2005).

Currently, some countries in locations with higher ethanol and fuel prices are producing ethanol from cellulosic feedstocks. It is only recently that cost-effective technologies for producing EFC in the US have started to emerge (Badger 2002). In Canada, Iogen Corporation built a small commercial-scale cellulose-ethanol plant using proprietary enzymatic hydrolysis technology. In 1997, they partnered with Petro-Canada to produce cellulose-ethanol beginning with a 1-million-gallon-per-year ethanol demonstration facility, located at Iogen's headquarters in Ottawa, using corn stover and switchgrass (Energy & Environmental Research Center, 2001). In summer 2005, a Swedish plant in Ömsköldsvik started to produce ethanol from sawdust. The production is still in a start-up phase, but the optimism is high. In a not so distant future, Sweden could become self-sufficient of ethanol from wood and wood residues, which would be a much more sustainable way of supplying ethanol to the Swedish market (Advanced course in LCA 2005).

Nowadays, in the field of sugar and starch utilization, the large-scale application of modern bioenergy conversion technologies has already occurred in a number of countries, both in the industrialized and developing worlds. In the US, the Minnesota Pollution Control Agency (MPCA) has scheduled a public information meeting in early 2005 to discuss the proposed Heron Lake BioEnergy ethanol project. The proposed plant would cover 37 acres at a site about 1 mile northeast of the city of Heron Lake in Jackson County. It would process 21.7 million bushels of corn annually to produce 55 million gallons of ethanol and 193,300 tons of distiller dried grains (Sullivan 2005). Another example is that of Brazil, a country that has committed itself to the development of its modern bioenergy

**Table 3** The lists of pilot plants for ethanol production from biomass

Year	Place	Substrates	Capacity (ton/day)	Production (l ethanol/day)	References
1976	US	—	1	—	Emert and Katzen 1980; Emert et al. 1980
1981	Canada	Grain	960	27,400–220,000	Robert 2004
1983	—	Cellulose	2,000	57,750	Emert et al. 1983
1983	Japan	—	720	150–200	Morikawa et al. 1985a,b; Morikawa and Tadokoro 1987
1984	Canada	—	1	—	Bente 1984
1988	France	Cellulose	96	160–190 kg/1,000 kg wood	Ballerini et al. 1994; Nativel et al. 1992
1993	US	Concentrated sweet whey	7.5	5,178	National Renewable Energy Laboratory 1996
2001	US	Corn	—	155,000	Gary 2002
2002	US	MSW	—	10,360,000	Badger 2002
2003	Canada	Lignocellulosic	—	41,500	Tembec 2003
2005	US	—	—	570,000	MN Pollution Control Agency 2005

potential. Its sugarcane-based ethanol industry annually produces around 15 billion liters from about 350 distilleries and satisfies over 33% of the country's gasoline needs (Agama Energy 2003).

For the Global ethanol market, Brazil has more than 300 plants, producing 15 billion liters per year and supplying 3 million cars with pure ethanol. In the US, there are more than 80 plants producing 10 billion liters per year, which it intends to increase to 19 billion liters by 2010. China could create 3 billion liters of ethanol per year. India's annual production of ethanol is 2.7 billion liters, and Eastern Europe's 2.5 billion liters. Western Europe's production ability is 2 billion liters and in Canada, 0.24 billion liters could be achieved and possibly expanded to 1.4 billion liters (Klein 2005).

Moreover, a fuel tax exemption is necessary for ethanol to compete with gasoline. Biodiesel from waste vegetable oil is already nearly competitive with conventional diesel, which cannot be said of biodiesel made from far more expensive virgin oils. It is foreseen that within the next 5–10 years, renewable, alternative transportation fuels from biomass and wastes will be competitive with fuels derived from petroleum at about US \$ 0.2 per liter.

Generally, economic restrictions force industrial processes to work in a very small range of operating conditions. For some batch processes which have long operating times in each cycle and depend strongly on the operating variables, it is very important to define the optimum conditions to achieve sufficient profitability. Kinetic models describing the behavior of microbiological systems can be a highly appreciated tool and can reduce tests to eliminate extreme possibilities.

### Most promising prospects

Ethanol fermentation involves significantly greater challenges, owing to the necessity of converting xylose as well as glucose to ethanol in the process, the microorganism–enzyme compatibility in SSF, and the low rates of cellulose hydrolysis. Recently, research has concentrated on the development of improved processes; however, there are still challenges that need further investigations.

#### Fermentation technology converting xylose to ethanol

Major fermentable sugars in hydrolyzate from cellulose and hemicellulose are glucose and xylose. Glucose fermentation to ethanol can be carried out efficiently by *S. cerevisiae*. In contrast, xylose fermentation is challenging because only a few traditional ethanol-producing microorganisms can readily ferment xylose, though many microorganisms utilize xylose as a carbon source. Efforts were made to improve ethanol fermentation from xylose (Jeffries and Shi 1999; Ho et al. 1999; Ingram et al. 1987; Zhang et al. 1995).

However, low ethanol yields, by-product formation, neutral pH requirement for growth, and intolerance to high

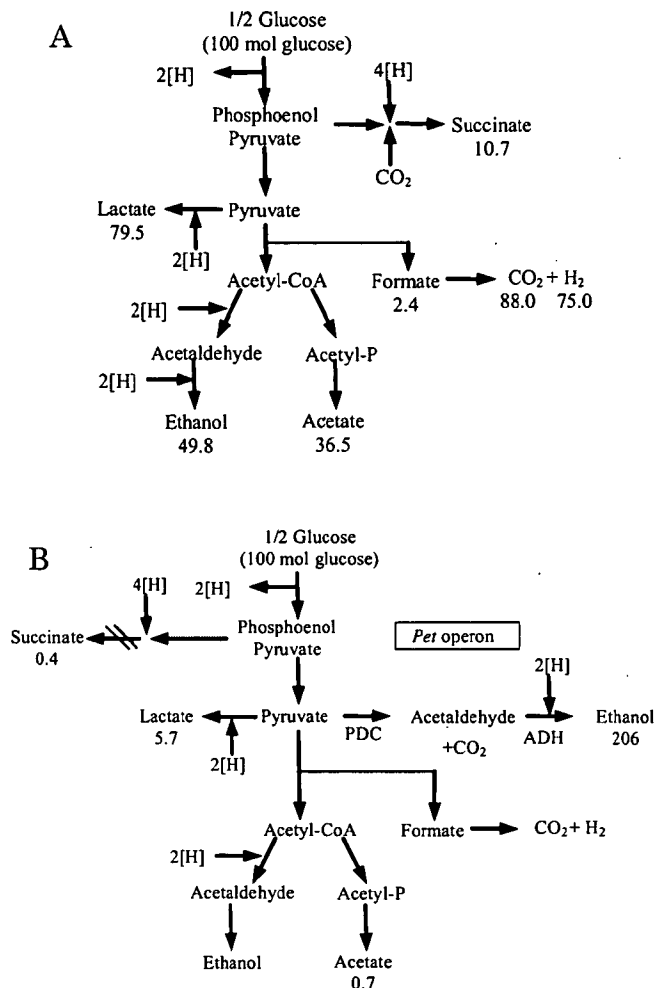
ethanol concentration are disadvantages in using bacteria in large-scale fermentation (Bothast et al. 1999). Currently, the bacterial conversion of xylose to ethanol has been studied mostly with utilizing the recombinant microorganisms.

The recombinant *E. coli* was used for ethanol production from xylose, and this ethanologenic strain (KO11) was able to convert glucose and xylose to ethanol at yields of 103–106% of theoretical value (Gonzalez et al. 2003; Tao et al. 2001). The extra ethanol was thought to arise from fermentation of carbohydrates present in the rich medium that was not accounted for in the sugar balance. Moreover, KO11 grows faster on xylose-containing medium than its parent strain ATCC11303. Comparison of global gene expression by microarray technology demonstrated that KO11 overexpresses xylose metabolism genes (Tao et al. 2001). During the combination, two genes are needed, one for pyruvate decarboxylase and another for alcohol dehydrogenase. These enzymes working together in the cell will divert pyruvate away from other fermentation products to ethanol. This would convert *E. coli* into an ethanologenic microorganism. The steps by initial *E. coli* and ethanologenic *E. coli* in alcoholic fermentation are shown in Fig. 3 (Gottschalk 1986; Matthew et al. 2005).

Similarly, the ethanol-producing bacterium *Z. mobilis* was metabolically engineered to broaden its range of fermentable substrates to include the pentose sugar xylose. Two operons encoding xylose assimilation and pentose phosphate pathway enzymes were constructed and transformed into *Z. mobilis*. The recombinant efficiently fermented both glucose and xylose, which is essential for economical conversion of lignocellulosic biomass to ethanol (Ingram and Doran 1995; Lynd et al. 2002; McMillan et al. 1999; Sun and Cheng 2002; Zhang et al. 1995). Currently, bacteria modified by this approach must operate at neutral pH where control of invasion by other organisms is more difficult than at the more acidic pH levels typical of most yeasts.

Moreover, Tolan and Finn (1987) transformed *Klebsiella planticola* ATCC 33531 with multicopy plasmids containing the *pdc* gene inserted from *Z. mobilis*, and expression of the gene markedly increased the yield of ethanol to 1.3 mol per mole of xylose, or 25.1 g/l. Concurrently, there was significant decrease in the yield of other organic by-products (i.e., formate, acetate, lactate and butanediol).

There have also been several yeast strains which were capable of fermenting xylose to produce ethanol in batch culture. Fein et al. (1984) isolated seven strains which were capable of fermenting xylose to produce ethanol from crude wood hydrolyzate in batch culture. Xylitol was found to be one of the major by-products, and the amount of xylitol varied depending on the strain used. *Candida tropicalis* showed the greatest potential for ethanol production from xylose. The crude acid hydrolyzate was inhibitory to all strains of yeast, even at dilute hydrolyzate concentrations. Strain acclimatization and chemical pretreatment resulted in a marked increase in utilization of substrates in acidic crude hydrolyzate. In an attempt to develop a xylose-fermenting yeast for industrial ethanol production, UV light-induced mutants of *Pachysolen*



**Fig. 3** a Typical fermentation products made by a K12 *Escherichia coli* fermenting glucose. Products are in moles produced per 100 mol fermented glucose (Dien et al. 2003; Gottschalk 1986) with 91% of the carbon accounted for as fermentation products. b Transforming *E. coli* with pet operon diverts almost all glucose to ethanol. This strain (KO11) also carries a mutation that blocks succinate production. Amount of each fermentation product is shown per 100 mol glucose (Dien et al. 2003; Ohta et al. 1991). Moles of CO<sub>2</sub> produced was not measured, but should be 206 mol based on ethanol production

*tannophilus* have been isolated, which can grow faster on xylose. Several other yeast strains for xylose utilization have been reported (Jeewon 1997).

On the other hand, *S. cerevisiae* traditionally has been used for ethanol production, such as beer and wine fermentation. This yeast does not exhibit many of the limitations encountered with bacteria. However, *S. cerevisiae* is not able to ferment xylose. Therefore, metabolic engineering of xylose fermentation in *S. cerevisiae* is an attractive approach (Sonderegger and Sauer 2003).

Although some significant progress can be noted in this field, there are still some problems which exist. One of them is ethanol inhibition. Ethanol inhibition of yeasts and other microorganisms has received much attention in microbial conversion of xylose to ethanol (Ghasem et al.

2004; Jeewon 1997; Palmqvist and Hahn-Hagerdal 2000). Xylose-fermenting yeasts do not grow under anoxic conditions and do not ferment when fully aerobic. Therefore, development of fermentation glucose and xylose efficiently is required for large-scale industrial application.

### Cellulase enzyme

Using lignocellulosic materials such as agricultural residues, grasses, forestry wastes, and other low-cost biomass can significantly reduce the cost of raw materials (compared to corn) for ethanol production. A reduction of the cost of ethanol production can be achieved by reducing the cost of either the raw materials or the cellulase enzymes. It was predicted that the use of genetically engineered raw materials with higher carbohydrate content combined with the improvement of conversion technology could reduce the cost of ethanol by US \$0.11 per liter over the next 10 years (Wooley et al. 1999).

Xylose metabolism employs pathways distinctly different from those involved in the utilization of glucose. With most yeast, xylose metabolism requires aerobic conditions at which cellular respiration is promoted; however, xylose is fermented to ethanol in poor yields and at low rates. To get around this problem, it has been proposed that the xylose fraction first be converted to readily fermentable xylulose, i.e., enzyme-mediated fermentation of xylose to ethanol using the bacterial enzyme, xylose isomerase.

Reducing the cost of cellulase enzyme production is a key issue in the enzymatic hydrolysis of lignocellulosic materials. Genetic techniques have been used to clone the cellulase coding sequences into bacteria, yeasts, fungi, and plants to create new cellulase production systems with possible improvement of enzyme production and activity. Riley et al. (1996) and Wood et al. (1997) reported the expression of recombinant endoglucanase genes from *Erwinia chrysanthemi* P86021 in *E. coli* KO11, and the recombinant system produced 3,200 IU endoglucanase/l fermentation broth (IU, international unit, defined as a micromole of reducing sugar as glucose released per minute using carboxymethyl cellulose as substrate). The thermostable endoglucanase E1 from *Acidothermus cellulolyticus* was expressed in *Arabidopsis thaliana* leaves (Ziegler et al. 2000), potato (Dai et al. 2000), and tobacco (Hooker et al. 2001).

### Immobilization

As in the case of microalgae culture in open ponds, microecological engineering techniques will need to be developed to maintain such strains in large systems which could be subject to invasion and contamination by potentially much faster growing wild microbes. Such microecological techniques would relieve the constraints of having to maximize the amounts and activities of the enzymes used in this process and/or maintain strictly aseptic conditions which are not economical. If intact mi-

crobial cells are directly immobilized, the removal of microorganisms from downstream product can be omitted, and the loss of intracellular enzyme activity can be kept to a minimum level (Najafpour 1990).

Use of biofilm reactors for ethanol production has been investigated to improve economics and the performance of fermentation processes (Vega et al. 1988). Immobilization of microbial cells for fermentation has been developed to eliminate inhibition caused by high concentration of substrate and product and also to enhance productivity and yield of ethanol. The work on ethanol production in an immobilized cell reactor (ICR) showed that production of ethanol using *Z. mobilis* was doubled (Ghasem et al. 2004; Takamitsu et al. 1993). The immobilized recombinant *Z. mobilis* was also successfully used with high concentrations of 12–15% sugar (Yamada et al. 2002).

Recently, immobilized biomass activity has been given more attention since it has been acknowledged to play a significant role in bioreactor performance (Gikas and Livingston 1997; Yamada et al. 2002). Frequently, immobilized cells are subjected to limitations in the supply of nutrients to the cells. Thus, because of the presence of heterogeneous materials such as immobilized cells, there is no convective flow inside the beads and the cells can receive nutrients only by diffusion (Riley et al. 1996). Immobilization of cells to a solid matrix is an alternative means of high biomass retention. The cells divide within and on the core of the matrix (Senthuran et al. 1997).

#### Simultaneous saccharification and fermentation

Simultaneous saccharification and fermentation (SSF) gives higher reported ethanol yields and requires lower amounts of enzyme because end-product inhibition from cellobiose and glucose formed during enzymatic hydrolysis is relieved by the yeast fermentation (Banat et al. 1998; McMillan et al. 1999). However, it is not easy to meet all the requirements of industry due to their low rates of cellulose hydrolysis, which is the stage limiting the rate of alcohol production. Another problem arises from the fact that most microorganisms used for converting cellulosic feedstock cannot utilize xylose, a hemicellulose hydrolysis product. Moreover, SSF requires that enzyme and culture conditions be compatible with respect to pH and temperature. *T. reesei* cellulases, which constitute the most active preparations, have optimal activity at pH 4.5 and 55°C. For *Saccharomyces* cultures, SSF are typically controlled at pH 4.5 and 37°C.

To overcome the problems related to SSF, many species of yeasts, as well as the bacterium *Z. mobilis*, have been tested with cellulases produced by *T. reesei* mutants (Chaudhuri and Sahai 1993; Haltrich et al. 1994; Spindler et al. 1992). The currently most promising ethanologenic bacteria for industrial exploitation are *E. coli*, *Klebsiella oxytoca*, and *Z. mobilis* (Matthew et al. 2005). Genetic engineering made it possible to transfer cellulose genes from *Trichoderma* to *S. cerevisiae* (Shoemaker 1984). However, the cellulases were produced at a concentration

too low to be useful. There is a group of microorganisms (*Clostridium*, *Cellulomonas*, *Trichoderma*, *Penicillium*, *Neurospora*, *Fusarium*, *Aspergillus*, etc.) showing a high cellulolytic and hemicellulolytic activity, which are also highly capable of fermenting monosaccharides to ethanol. It may be possible, within this group of microorganisms, to produce “superstrains” via genetic engineering capable of hydrolyzing cellulose and xylan along with fermentation of glucose and xylose to ethanol.

Moreover, to make the SSF process more effective, it has also been found necessary to search for thermostable strains capable of producing substantial amounts of ethyl alcohol at temperatures optimal for saccharification and suitably resistant to ethanol (Szczodrak and Targonski 1988).

Roychoudhury et al. (1992) have developed a notable way of eliminating the negative effects which excessive concentrations of ethanol have on yeast activity and cellulase within the SSF system. They used a vacuum cycling reactor where the concentration of ethanol was kept at a relatively low level by its removal from the flash chamber.

However, more efforts have to be made in the development of microorganisms for industrial ethanol production. In addition, it is important to keep the rate-limiting step in mind. In SSF, the ethanol production rate is controlled by the cellulase hydrolysis rate and not the glucose fermentation, and hence, steps to increase the rate of hydrolysis will lower the cost of ethanol production via SSF.

#### Sugar conversion

Since sugars are already available in a degradable form and yeast cells can metabolize sugars directly, these substrates require the least costly preparation. The other carbohydrates must be hydrolyzed to sugars before they can be metabolized. Several studies have dealt with the economic assessment of using cellulose hydrolysate, either from waste (Cysewski and Wilke 1976; Green et al. 1989; Maiorella et al. 1984; Wilke et al. 1976) or from wood (Hinman et al. 1992; Marco et al. 2002). One disadvantage with the application of these materials is their low sugar content resulting in low cell and ethanol concentrations. Hence, although starchy or cellulosic materials are cheaper than sugar-containing raw materials, the requirement for converting the starchy or cellulosic materials to fermentable sugars is a disadvantage of these substrates (Lynd et al. 2001).

Moreover, microorganisms used in industry are selected to provide the best possible combination of characteristics for the process and equipment being used. The selected strains should have tolerance to high concentrations of sugar and ethanol (Keim 1983; Oh et al. 2000).

Ethanol inhibition of yeasts and other microorganisms has received much attention (Casey and Ingledew 1986) in microbial production of ethanol. Lucas and van Uden (1985) investigated the effects of temperature on ethanol tolerance and thermal death of *Candida shehatae* and

determined that it was more tolerant of ethanol at lower temperatures. Du Preez et al. (1987) quantitatively evaluated the effects of ethanol on the growth of the xylose-fermenting yeasts *C. shehatae* and *Pichia stipitis* using Luong kinetics. The effect of ethanol on metabolic rate has been examined with ethanol added exogenously. Both Lucas and van Uden (1985) and du Preez et al. (1987) placed cells into media containing different concentrations of ethanol and measured the specific growth rate which ensued. Unfortunately, less inhibition is observed with exogenous ethanol than with the same concentration of ethanol produced endogenously (Hoppe and Hansford 1982; Novak et al. 1981; du Preez et al. 1987). Some have claimed that the apparently greater inhibition by endogenously produced ethanol reflects the tendency of actively fermenting cells to accumulate ethanol intracellularly (Casey and Ingledew 1986; Ghasem et al. 2004); however, the yeast plasma membrane is known to be very permeable to ethanol, which casts doubt on this hypothesis. Whatever the reason for the different effects of externally added and internally generated ethanol, realistic assessments of ethanol inhibition ought to involve ethanol generated in situ.

On the other hand, since the distillation cost per unit amount of ethanol produced is substantially higher at low ethanol concentrations (Zacchi and Axelsson 1989), several investigators have dealt with the idea of concentrating sugar solutions prior to fermentation (Cysewski et al. 1976; Iraj et al. 2002; Oh et al. 2000; Zacchi and Axelsson 1989). Clearly, it is necessary to solve the problem between the concentration of ethanol produced and sugar added if an economically sustainable system is to be created using this method.

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## Metabolic engineering for improved fermentation of pentoses by yeasts

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**Abstract** The fermentation of xylose is essential for the bioconversion of lignocellulose to fuels and chemicals, but wild-type strains of *Saccharomyces cerevisiae* do not metabolize xylose, so researchers have engineered xylose metabolism in this yeast. Glucose transporters mediate xylose uptake, but no transporter specific for xylose has yet been identified. Over-expressing genes for aldose (xylose) reductase, xylitol dehydrogenase and moderate levels of xylulokinase enable xylose assimilation and fermentation, but a balanced supply of NAD(P) and NAD(P)H must be maintained to avoid xylitol production. Reducing production of NADPH by blocking the oxidative pentose phosphate cycle can reduce xylitol formation, but this occurs at the expense of xylose assimilation. Respiration is critical for growth on xylose by both native xylose-fermenting yeasts and recombinant *S. cerevisiae*. Anaerobic growth by recombinant mutants has been reported. Reducing the respiration capacity of xylose-metabolizing yeasts increases ethanol production. Recently, two routes for arabinose metabolism have been engineered in *S. cerevisiae* and adapted strains of *Pichia stipitis* have been shown to ferment hydrolysates with ethanol yields of 0.45 g g<sup>-1</sup> sugar consumed, so commercialization seems feasible for some applications.

### Introduction

Fermentation of xylose to ethanol is driven by political, economic and technical considerations. For example, using biomass as a feedstock for renewable fuel production can substantially reduce the accumulation of green-

house gasses (McMillan 1997; Claassen et al. 1999; Wyman 1999; Kheshgi et al. 2000), so a recent European Union directive proposed that biofuels should represent 2% of total transportation fuel consumption by 2005 and 5.75% by 2010 (Roca and Olsson 2003). Increased domestic use of agricultural commodities can increase income for farmers, so the agricultural policies of the United States, Brazil and the European Union created nascent ethanol industries for the commercial production of ethanol from grains, sugar cane and other feedstocks. Opportunities for the bioconversion of harvest and processing residues are increasing along with these markets. For example, as grain hull, corn cobs, corn stover and sugar cane bagasse byproducts increase, ethanol production from the waste streams becomes feasible (Saha et al. 1998; Saha and Bothast 1999). Xylose is a major constituent of these and other renewable biomass feedstocks, but its efficient utilization—which is essential for commercial bioconversion (Hinmann et al. 1989; Olsson and Hahn-Hägerdal 1996)—presents a technical barrier. Lignocellulosic crop residues comprise more than half of the world's agricultural phytomass (Smil 1999) and significant fractions of the total can be recovered without competing with other uses (Lynd 1996; Wyman 1999). Xylose constitutes about 17% of the total dry weight in woody angiosperms and ranges up to 31% in herbaceous angiosperms (Pettersen 1984; Hespell 1998). One source of xylose is the sulfite-pulping of hardwood (Lawford and Rousseau 1993). Depending on the substrate and reaction conditions, dilute acid pretreatments of lignocellulosic residues can recover 80–95% of the xylose from the feedstock (Chen et al. 1998; Kim et al. 2001; Aguilar et al. 2002).

Native strains of *Saccharomyces cerevisiae* do not use xylose as a carbon source. *Candida utilis* or "torula yeast" will grow on xylose, but this yeast is strictly aerobic and does not produce ethanol. In the early 1980s, following the discovery that *S. cerevisiae*, *Schizosaccharomyces pombe* and other yeasts can ferment D-xylulose to ethanol (Wang and Schneider 1980; Wang et al. 1980), intensive screening efforts rapidly revealed that some can convert xylose

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to ethanol directly under aerobic or oxygen-limiting conditions (Schneider et al. 1981; Jeffries 1982; Slininger et al. 1982). Attention focused on *Pachysolen tannophilus*, *C. shehatae* (Du Preez and van der Walt 1983) and *Pichia stipitis*, which are the best native xylose-fermenting yeasts known (Toivola et al. 1984; Du Preez et al. 1986). Many improvements have been made in the genetic engineering of yeasts and bacteria for the fermentation of xylose and arabinose to ethanol and other products such as lactic acid. However, the bioconversion of pentoses to ethanol still presents a considerable economic and technical challenge (Jeffries and Shi 1999; Aristidou and Penttilä 2000; Hahn-Hägerdal et al. 2001).

The objective of this review is to assess the current state of microbial strain development for the fermentation of pentose sugars. This is a very active field. From January 1999 to June 2003, more than 60 articles on yeast appeared in press. We try to emphasize the latest work and refer the reader to reviews of the earlier literature (Jeffries 1983, 1985; Prior et al. 1989; Jeffries and Kurtzman 1994; Gong et al. 1999; Ho et al. 1999; Jeffries and Shi 1999; Aristidou and Penttilä 2000; Flores et al. 2000; Jeffries and Jin 2000; Ostergaard et al. 2000; Dequin 2001; Hahn-Hägerdal et al. 2001; Galbe and Zacchi 2002).

## Metabolic engineering of yeasts

Yeast strain development focuses on the genetic engineering of *Saccharomyces cerevisiae*, but *P. stipitis* has also been modified for xylose fermentation. Metabolic engineering can alter sugar transport, assimilation, the pentose phosphate pathway, glycolysis, the terminal steps of fermentation and the relatively complicated interplay between respiration and fermentation that determine the intracellular redox balance. While a few of the changes enable xylose utilization in *S. cerevisiae*, most have marginal effects. No one factor or enzymatic step is rate-limiting, but some are critical. Even though genes for xylose assimilation are present in *S. cerevisiae*, they are not expressed at a sufficient level to enable significant sugar assimilation. Engineering of *P. stipitis* has been more limited, but some principles have been established with this yeast.

## Xylose transport

Sugar uptake limits xylose utilization in both *S. cerevisiae* and *P. stipitis*. In *S. cerevisiae*, the *HXT* family of sugar transporters mediates glucose uptake (Kruckeberg 1996; Boles and Hollenberg 1997). *Hxt1*–*Hxt7* and *Gal2* exhibit counter-transport when individually expressed in a *hxt1-7* null mutant of *S. cerevisiae*, which indicates that they function by facilitated diffusion (Maier et al. 2002). Kinetic studies with  $^{14}\text{C}$  glucose can distinguish high- and low-affinity uptake systems. Xylose uptake competes with glucose uptake, indicating that they share transport components (Meinander and Hahn-Hägerdal 1997). *S.*

*cerevisiae* takes up xylose by both low- and high-affinity glucose transport systems (Lee et al. 2002), but after incubation with xylose only the high-affinity system is detected. Cultivation of the xylose-fermenting *S. cerevisiae* FPL-YSX3 on xylose under aerobic or oxygen-limiting conditions strongly induces (5- to 50-fold) the high-affinity transporters *HXT2*, *HXT6* and *HXT7* and the moderate affinity transporter *HXT5* (Buziol et al. 2002; Jin 2002). *Hxt5* is produced under slow growth conditions (Diderich et al. 2001; Verwaal et al. 2002). With YSX3 cells cultivated in xylose medium, the low-affinity transporters *HXT1* and *HXT3* are expressed at 2–5% of the level observed with cells grown on glucose (Jin 2002). These results suggest that engineered *S. cerevisiae* mainly uses the high-affinity system for xylose transport.

Glucose strongly inhibits the transport of xylose by both high- and low-affinity systems (van Zyl et al. 1993). The native *S. cerevisiae* glucose transporters exhibit a significantly lower affinity for xylose ( $K_m=49$ –300 mM) than for glucose ( $K_m=1$ –28 mM; Kötter and Ciriacy 1993; Lagunas 1993; Van Zyl et al. 1999). Therefore, glucose and xylose are consumed simultaneously only under glucose-limited conditions (Meinander and Hahn-Hägerdal 1997). *S. cerevisiae* (TMB3201)—which is completely deficient in all 18 monosaccharide transporters (Wieczorke et al. 1999) but has a functional xylose utilization pathway (Hamacher et al. 2002)—cannot take up or grow on xylose. When various *HXT* genes are reintroduced, *Hxt4*, *Hxt5*, *Hxt7* and *Gal2* promote xylose uptake (Hamacher et al. 2002).

Native xylose-metabolizing yeasts have two kinetically distinct xylose transport systems. The low-affinity system is shared with glucose, while the high-affinity system is specific for xylose (Hahn-Hägerdal et al. 2001). Both of these systems are tightly coupled to energy metabolism (Kilian and van Uden 1988; Does and Bisson 1989). Three genes, *SUT1*, *SUT2* and *SUT3*, encode glucose transporters in *P. stipitis* (Weierstall et al. 1999). *Sut2* and *Sut3* are highly similar to the *S. cerevisiae* glucose transporter family and the *Sut2* and *Sut3* transporters have a higher affinity for glucose than for xylose. Transcription of *SUT1* is induced in *P. stipitis* independently of the oxygen supply. *SUT2* and *SUT3* are expressed only under aerobic conditions, but independently of the carbon source. Disruption of *SUT1* eliminates the low-affinity xylose transport system in *P. stipitis*. Xylose uptake in *P. stipitis* grown on glucose has high-affinity ( $K_{m1}=3.2$  mM) and low-affinity ( $K_{m2}=80$  mM) components. In  $\Delta sut1$  cells grown under oxygen-limiting conditions—when *SUT2* and *SUT3* are not expressed—xylose transport is still active, which suggests that specific xylose transporters other than *Sut1*, *Sut2* and *Sut3* are present. However, Weierstall et al. (1999) were not able to identify any additional cross-hybridization signals for *HXT*- or *SUT*-related genes in *P. stipitis*.

## Xylose isomerase

The initial metabolic engineering of *S. cerevisiae* for xylose assimilation attempted the heterologous expression of bacterial xylose isomerase (XI). This approach was reasonable, given that *S. cerevisiae* can grow on and ferment xylulose, but significant XI activity was not attained in the transformed cells (Jeffries and Shi 1999; Hahn-Hägerdal et al. 2001). Probably, this is attributable to improper folding of the protein (Sarthy et al. 1987), but even if small amounts of active XI are formed, xylitol is a competitive inhibitor (Smith et al. 1991). Expression of a XI from the thermophilic bacterium *Thermus thermophilus* achieved the heterologous production of an active enzyme in *S. cerevisiae* (Walfridsson et al. 1996) and the genetic background has been modified to reduce xylitol production (Walfridsson et al. 1996; Träff et al. 2001). However, the temperature required for moderate *T. thermophilus* XI activity is well above the maximum growth temperature of *Saccharomyces*, so researchers from this same laboratory have used directed evolution to obtain a XI with 9-fold higher activity constants at 60°C and much higher inhibition constants for xylitol (Lönn et al. 2002). Very recently, Harhangi et al. (2003) introduced a eukaryotic XI, the *AraA* gene, from the anaerobic fungus species into *S. cerevisiae* and enabled slow xylose assimilation by this route (Kuyper et al. 2003). Whether the use of XI will prove successful could depend on other factors. At equilibrium, energetics of the isomerization between xylose and xylulose favors xylose formation by 83:17 (Jeffries 1985), so some other driving force is necessary to promote this reaction.

## Xylose reductase and xylitol dehydrogenase

As early as 1983, researchers concluded that the key to anaerobic assimilation of xylose by native yeasts such as *Pachystolon tannophilus* was the presence of an aldose (xylose) reductase (XR) that could accept either NADH or NADPH as a cofactor (Bruinenberg et al. 1983a, 1983b, 1984; Verduyn et al. 1985a, 1985b; Bruinenberg 1986). This hypothesis was based on a study of aerobic xylose utilization by *C. utilis*. Even though this yeast can rapidly ferment glucose, fermentative activity ceases immediately after transfer to xylose. The XR of *C. utilis* exclusively uses NADH as a cofactor. In comparison, at least one XR of *P. tannophilus* can use either NADH or NADPH (Verduyn et al. 1985a). The same is true of *Pichia stipitis* (Verduyn et al. 1985b) and *C. shehatae* (Ho et al. 1990); and all of these can metabolize xylose anaerobically—even though they do not grow under those conditions (Wijsman et al. 1985). Because the assimilation of xylose requires two oxidoreductase steps and because all oxidoreductase reactions following these are balanced, researchers hypothesized that, if no transhydrogenase were present to regenerate NAD<sup>+</sup> and NADPH, xylose assimilation under anaerobic conditions would quickly halt.

When the XR of *P. stipitis* is cloned and expressed in *S. cerevisiae* (Amore et al. 1991; Takuma et al. 1991; Tantirungkij et al. 1993, 1994; Billard et al. 1995; Handumrongkul et al. 1998), the resulting transformants produce xylitol if some other carbon source is present to provide a reductant (Hallborn et al. 1991, 1994). When glucose is used as a co-substrate, xylose assimilation and xylitol production are reduced, presumably because of competition for transport (Thestrup and Hahn-Hägerdal 1995). *XYL1* does not limit xylose assimilation in *P. stipitis* (Dahn et al. 1996). To produce ethanol, it is necessary to have a system that can oxidize xylitol to xylulose while reducing acetaldehyde under oxygen-limiting conditions.

Isolation of the first two genes for xylose assimilation from *P. stipitis* led to the initial development of *S. cerevisiae* strains able to metabolize xylose (Kötter et al. 1990; Amore et al. 1991). *S. cerevisiae* transformed with these two genes can use xylose oxidatively and produce xylitol without the addition of a co-metabolizable carbon source. Increasing xylitol dehydrogenase (XDH) activity relative to XR (XR:XDH=0.6) produces less xylitol and more ethanol than when XR is present in greater abundance (Walfridsson et al. 1997). When *XYL2* is strongly expressed, xylulose is secreted—indicating that xylulokinase (XK) activity limits xylose metabolism in these cells (Jin and Jeffries 2003).

## Xylulokinase

Much has been reported about *S. cerevisiae* engineered to express XK. Chang and Ho cloned D-xylulokinase from *Pachysolen tannophilus* and *S. cerevisiae* as early as 1988 (Chang and Ho 1988; Deng and Ho 1990) and were the first to report a sequence for the *S. cerevisiae* XK gene in a patent (Ho and Tsao 1993). The complete *S. cerevisiae* gene, *XKS1*, was obtained in the yeast genome project (Rodriguez-Pena et al. 1998). The protein coded for by the original Ho and Tsao sequence has been reported as inactive (Eliasson et al. 2000a). D-xylulokinase activity limits the metabolism of D-xylulose in *S. cerevisiae* (Chang and Ho 1988; Deng and Ho 1990) when *S. cerevisiae* xylulokinase is overexpressed along with *Pichia stipitis* *XYL1* and *XYL2* in *Saccharomyces* sp. 1400, which is a fusant product of *S. diastolicus* and *S. uvarum* (Moniruzzaman et al. 1997; Ho et al. 1998). *Saccharomyces* sp. 1400 (pLNH33) can ferment a mixture of 53 g glucose l<sup>-1</sup> and 56 g xylose l<sup>-1</sup> to give an ethanol concentration of 50 g l<sup>-1</sup> within 36 h. (Krishnan et al. 1999). This is the highest ethanol yield and fermentation rate from glucose/xylose mixtures reported for a recombinant *Saccharomyces* to date.

*S. cerevisiae* CEN.PK 113-7A, a strain with a well defined genetic background, has also been developed as a xylose-fermenting yeast. *XYL1*, *XYL2* and *XKS1* were each integrated into the chromosome under the control of the *S. cerevisiae* PGK1 promoter to obtain a stable transformant designated *S. cerevisiae* TMB3001 (Eliasson et al. 2000b).

Table 1 Kinetic parameters for xylose fermentation by yeasts

Species/strain	Description	Conditions	Carbon source	Yield (g g <sup>-1</sup> )		Ethanol productivity (g l <sup>-1</sup> ·h <sup>-1</sup> )	Reference
				Ethanol	Xylitol		
<i>Pichia stipitis</i>							
CBS 7126	Wild type	Respiro-fermentative	Xylose (50 g l <sup>-1</sup> )	0.43	0	0.12	(Du Preez et al. 1986)
FPL-UC7	Parental strain of Shi21	Fermentative	Xylose (80 g l <sup>-1</sup> ) in complex medium	0.38	0	0.17	(Shi et al. 1999)
FPL-Shi21	Disruption of <i>PSCYC1</i>	Fermentative	Xylose (80 g l <sup>-1</sup> ) in complex medium	0.46	0	0.09	(Shi et al. 1999)
FPL-UC7	Parental strain of Shi31	Respiro-fermentative	Xylose (80 g l <sup>-1</sup> ) in complex medium	0.25	0.03	0.16	(Shi et al. 2002)
FPL-Shi31	Disruption of <i>PSAOX1</i>	Respiro-fermentative	Xylose (80 g l <sup>-1</sup> ) in complex medium	0.31	0.03	0.13	(Shi et al. 2002)
<i>Saccharomyces cerevisiae</i>							
1400(pLNH32)	<i>XYL1, XYL2, XKS1</i>	Fermentative	Glucose (90 g l <sup>-1</sup> ) and xylose (40 g l <sup>-1</sup> ) in complex medium	0.46	0.17	1.15	(Ho et al. 1998)
1400(pLNH32)	<i>XYL1, XYL2, XKS1</i>	Fermentative	Xylose (50 g l <sup>-1</sup> ) in complex medium	0.3	0.08	0.33	(Ho et al. 1998)
TMB3001	<i>XYL1, XYL2, XKS1</i>	Aerobic batch fermentation	Glucose (50 g l <sup>-1</sup> ) and xylose (50 g l <sup>-1</sup> ) in minimal medium	0.23	0.08	0.034	(Zaldivar et al. 2002)
A	Industrial strain	Aerobic batch fermentation	Glucose (50 g l <sup>-1</sup> ) and xylose (50 g l <sup>-1</sup> ) in minimal medium	0.42	0.09	0.11	(Zaldivar et al. 2002)
A4	<i>XYL1, XYL2, XKS1</i> in A4	Aerobic batch fermentation	Glucose (50 g l <sup>-1</sup> ) and Xylose (50 g l <sup>-1</sup> ) in minimal medium	0.27	0.27	0.04	(Zaldivar et al. 2002)
A6	<i>XYL1, XYL2, XKS1</i> in A4	Aerobic batch fermentation	Glucose (50 g l <sup>-1</sup> ) and xylose (50 g l <sup>-1</sup> ) in minimal medium	0.27	0.32	0.046	(Zaldivar et al. 2002)
TMB3001	<i>XYL1, XYL2, XKS1</i>	Anaerobic continuous culture	Glucose (20 g l <sup>-1</sup> ) in minimal medium	0.30 (0.41)		0.084	(Eliasson et al. 2000b)
		Anaerobic continuous culture	Glucose (15 g l <sup>-1</sup> ) and xylose (5 g l <sup>-1</sup> ) in minimal medium	0.30 (0.39)	0.03	0.077	(Eliasson et al. 2000b)
		Anaerobic continuous culture	Glucose (10 g l <sup>-1</sup> ) and xylose (10 g l <sup>-1</sup> ) in minimal medium	0.29 (0.36)	0.09	0.089	(Eliasson et al. 2000b)
		Anaerobic continuous culture	Glucose (5 g l <sup>-1</sup> ) and xylose (15 g l <sup>-1</sup> ) in minimal medium	0.26 (0.35)	0.12	0.088	(Eliasson et al. 2000b)
H1691	<i>XYL1, XYL2, XKS1</i>	Anaerobic batch fermentation	Xylose (50 g l <sup>-1</sup> ) in minimal medium	0.09	0.41	0	(Toivari et al. 2001)
		Micro-aerobic batch fermentation	Xylose (50 g l <sup>-1</sup> ) in minimal medium	0.12	0.42	0.03	(Toivari et al. 2001)
H158	<i>XYL1, XYL2, XKS1</i>	Aerobic batch fermentation	Xylose (50 g l <sup>-1</sup> ) in minimal medium	0.06	0.06	0.16	(Toivari et al. 2001)
		Anaerobic batch culture	Xylose (80 g l <sup>-1</sup> ) in minimal medium	0	0.53	0	(van Zyl et al. 1999)
			Xylose (80 g l <sup>-1</sup> ) in complex medium	0	0.66	0	(van Zyl et al. 1999)
			Xylose (50 g l <sup>-1</sup> ) and glucose (20 g l <sup>-1</sup> ) in complex medium	0.1	0.61	0.07	(van Zyl et al. 1999)
			Xylose (50 g l <sup>-1</sup> ) and raffinose (20 g l <sup>-1</sup> ) in complex medium	0.08	0.67	0.06	(van Zyl et al. 1999)
H158-pXks	<i>XYL1, XYL2, XKS1</i>	Fermentative	Xylose (80 g l <sup>-1</sup> ) in defined medium	0.15	0	0.02	(Johansson et al. 2001)
			Xylose (80 g l <sup>-1</sup> ) in complex medium	0.20	0.03	0.03	(Johansson et al. 2001)
CEN.PK-pXks			Xylose (80 g l <sup>-1</sup> ) in defined medium	0.13	0.25	0.03	(Johansson et al. 2001)



Table 1 (continued)

Species/strain	Description	Conditions	Carbon source	Yield (g g <sup>-1</sup> )		Ethanol productivity (g l <sup>-1</sup> h <sup>-1</sup> )	Reference
				Ethanol	Xylitol	Biomass	
TMB3101	xylA in CEN.PK2-1C	Anaerobic batch culture	Xylose (80 g l <sup>-1</sup> ) in complex medium	0.17	0.36	0.04	(Johansson et al. 2001)
TMB3103	xylA, XKS1	Anaerobic batch culture	Xylose (50 g l <sup>-1</sup> ) in minimal medium	0	0.57	0	(Träff et al. 2001)
TMB3102	xylA with <i>GRE3</i> knockout	Anaerobic batch culture	Xylose (50 g l <sup>-1</sup> ) in minimal medium	0	0.52	0	(Träff et al. 2001)
TMB3104	xylA, XKS1 with <i>GRE3</i> knockout	Anaerobic batch culture	Xylose (50 g l <sup>-1</sup> ) in minimal medium	0.22	0.31	0.15	(Träff et al. 2001)
TMB3001	<i>XYL1</i> , <i>XYL2</i> , <i>XKS1</i>	Anaerobic batch culture	Xylose (50 g l <sup>-1</sup> ) in minimal medium	0	0.31	0	(Träff et al. 2001)
	Anaerobic continuous culture ( <i>D</i> =0.06 h <sup>-1</sup> )		Xylose (50 g l <sup>-1</sup> ) in minimal medium	0.31	0.29	0.20	(Jeppsson et al. 2002)
	Anaerobic continuous culture ( <i>D</i> =0.12 h <sup>-1</sup> )		Glucose (20 g l <sup>-1</sup> ) and Xylose (20 g/L) in minimal medium	0.30	0.43		(Jeppsson et al. 2002)
	Anaerobic batch culture		Glucose (20 g l <sup>-1</sup> ) and xylose (20 g l <sup>-1</sup> ) in minimal medium	0.31	0.37		(Jeppsson et al. 2002)
TMB3255	<i>ZWF1</i> knockout in TMB3001	Anaerobic batch culture	Xylose (50 g l <sup>-1</sup> ) in minimal medium	0.41	0.05	0.04	(Jeppsson et al. 2002)
	Anaerobic continuous culture ( <i>D</i> =0.06 h <sup>-1</sup> )		Glucose (20 g l <sup>-1</sup> ) and xylose (20 g l <sup>-1</sup> ) in minimal medium	0.39	0.13		(Jeppsson et al. 2002)
	Anaerobic continuous culture ( <i>D</i> =0.12 h <sup>-1</sup> )		Glucose (20 g l <sup>-1</sup> ) and xylose (20 g l <sup>-1</sup> ) in minimal medium	0.366	0.131		(Jeppsson et al. 2002)
FPL-YSX3	<i>XYL1</i> , <i>XYL2</i> , <i>XYL3</i>	Aerobic fermentation	Xylose (40 g l <sup>-1</sup> ) in minimal medium	0.09	0.33	0.07	(Jin et al. 2003)
FPL-YSX3	<i>XYL1</i> , <i>XYL2</i> , <i>XYL3</i>	Oxygen-limited fermentation	Xylose (40 g l <sup>-1</sup> ) in complex medium	0.12	0.55	0.06	(Jin 2002)
FPL-YSX3P	Respiration-deficient mutant of FPL-YSX3	Oxygen-limited fermentation	Xylose (40 g l <sup>-1</sup> ) in complex medium	0.29	0.46	0.11	(Jin 2002)

which, like *S. cerevisiae* sp. 1400, is able to ferment mixtures of glucose and xylose to ethanol, albeit with slightly lower yields (cf. Ho et al. 1999; Krishnan et al. 1999). Overexpression of *XKS1* clearly enhances xylose utilization under aerobic conditions, but xylose utilization declines by almost an order of magnitude with decreased aeration.

Intracellular ATP levels and the ratio of ATP:ADP are also dramatically lower in the recombinant yeast (Toivari et al. 2001). Overexpression of *XKS1*, *XYL1* and *XYL2* increased ethanol yield in two different strains of *S. cerevisiae*, but it also decreased total xylose consumption by 50–80% (Johansson et al. 2001; Table 1).

D-xylulokinase is not expressed significantly in native *S. cerevisiae* (Deng and Ho 1990), but it is essential for xylulose metabolism because *xks1* mutants do not grow on xylulose. At the same time, overexpression of *XKS1* can decrease growth on xylulose (Rodriguez-Pena et al. 1998). Richard et al. (2000) and later Johansson et al. (2001) suggested that overexpression of *XKS* combined with unlimited access to xylulose might lead to toxicity, due to ATP depletion in a manner similar to that observed with unregulated glucose uptake in *Saccharomyces tps1/ggs1* mutants (Hohmann et al. 1993, 1996; Thevelein and Hohmann 1995; Teusink et al. 1998). Jin et al. (2002) expressed the *P. stipitis* gene for D-xylulokinase, *XYL3*, in a *S. cerevisiae* strain that was engineered for high levels of *XYL1* and *XYL2* (Jin et al. 2003). The *XYL3* XK gene product is more specific for D-xylulose; and it has much lower activity against D-ribulose than the *S. cerevisiae* XK (Richard et al. 2000). When the *P. stipitis* XK activity is low, cell growth on xylose is not significantly affected. When *P. stipitis* XK is strongly overexpressed, aerobic growth is significantly inhibited on xylose. Aeration increases the toxicity of *XYL3* or *XKS1* overexpression. Xylulokinase activity is higher in cells grown on glucose,

but no growth inhibition is observed, which indicates that the inhibitory effect is specific to xylose uptake. Ethanol production and growth are optimal when low levels of *XYL3* are expressed from the native promoter. This suggests that the effect of overexpressing XK when D-xylulose is fully accessible is similar to substrate-accelerated cell death.

Deletion of *XKS1* completely blocks xylitol formation from xylose, but leads to arabitol accumulation and increases ethanol production (Eliasson et al. 2000a). Deletions within the *PGII* promoter that reduce phosphoglucose isomerase activity by one to two orders of magnitude increase the accumulation of fructose-6-phosphate and result in about 15% higher ethanol yield. *tps1* and *tps2* mutants accumulate sugar phosphates and increase ethanol yield by 20–30%. *gnd1* mutants show 30% higher ethanol yield, but *rpe1* mutants hardly assimilate xylulose at all—as one might expect, because both ribulose-5-phosphate and xylulose-5-phosphate are necessary to form ribose-5-phosphate and the other intermediates of the pentose phosphate pathway (PPP; Fig. 1).

While we can learn a great deal about the mechanics of xylose fermentation by genetically manipulating laboratory strains, commercialization of the xylose fermentation will necessitate the use of strains that can grow vigorously, ferment rapidly and tolerate acids or other inhibitory compounds (Hahn-Hägerdal et al. 2001). Many industrial *Saccharomyces* strains have been developed that have these characteristics. In fermenting a mixture of glucose and xylose (50 g l<sup>-1</sup> each), two industrial yeast strains expressing the xylose assimilation pathway produced more ethanol and consumed more xylose than an engineered laboratory strain (TMB 3001). However, the differences in ethanol production arose almost entirely

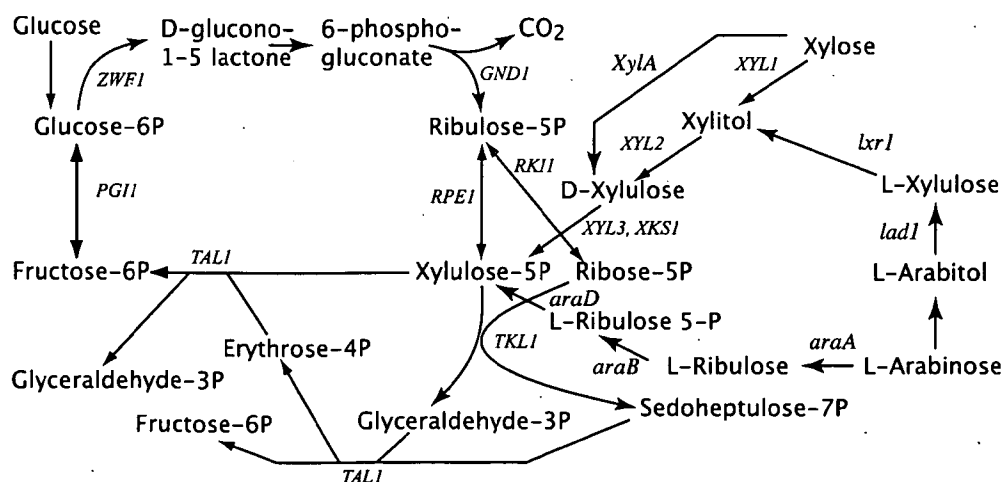


Fig. 1 The pentose phosphate cycle in *Saccharomyces cerevisiae* engineered for xylose and arabinose assimilation. Reduction of L-arabinose to L-arabitol is mediated by aldose reductase, *araA* *Bacillus subtilis*-arabinose isomerase, *araB*-ribulokinase, *araD*-ribulose-5-phosphate 4-epimerase, *GND1* *S. cerevisiae* phosphogluconate dehydrogenase, *lad1* *Trichoderma reesei*-arabinol 4-dehydrogenase, *lxr1* *T. reesei*-xylulose reductase, *PGII* *S. cerevisiae*

glucose-6-phosphate isomerase, *RKI1* *S. cerevisiae* ribose-5-phosphate isomerase, *RPE1* *S. cerevisiae* ribulose-phosphate 3-epimerase, *TAL1* *S. cerevisiae* transaldolase, *TKL1*, *S. cerevisiae* transketolase, *XKS1* *S. cerevisiae*-xylulokinase, *XYL1* *Pichia stipitis* xylose (aldose) reductase, *XYL2* *P. stipitis* xylitol dehydrogenase, *XYL3*, *P. stipitis*-xylulokinase, *XylA* *Piromyces* xylose isomerase, *ZWF1* *S. cerevisiae* glucose-6-phosphate 1-dehydrogenase

during glucose consumption rather than during the xylose consumption phase (Zaldivar et al. 2002).

### Pentose phosphate pathway

Kötter and Ciriacy hypothesized that the excessive production of xylitol by *S. cerevisiae* genetically engineered with *XYL1* and *XYL2* was limited by the dual cofactor capacity of the *P. stipitis* XR, by excessive activity of the oxidative PPP in *S. cerevisiae* and by insufficient capacity of the non-oxidative PPP (Kötter and Ciriacy 1993). Overexpression of the *P. stipitis* gene for transketolase (*TKL1*) in a *S. cerevisiae* strain expressing heterologous *XYL1* and *XYL2* greatly reduced growth of the transformant on xylose minimal medium (Metzger and Hollenberg 1994). Strains overexpressing the *P. stipitis* gene for transaldolase (*TAL1*), *XYL1* and *XYL2* grew faster than strains expressing *XYL1* and *XYL2* alone (Walfridsson et al. 1995). However, the plasmid burden due to the overexpression of *TAL1*, *XYL1* and *XYL2* reduced the growth rate of the transformant relative to the host strain (Bao et al. 1997; Meinander et al. 1999).

### Redox balance

The production of xylitol by recombinant *S. cerevisiae* is thought to originate from an overabundance of NADPH relative to NADH for the initial xylose assimilation step. To reduce xylitol production and thereby increase ethanol yield, Jeppson et al. (2002) overexpressed *XKS1* in *S. cerevisiae* *gnd1* and *zwf1* backgrounds. The  $\Delta zwf1$  mutant greatly increased ethanol yield by producing 0.41 g ethanol g<sup>-1</sup> xylose consumed, as compared with 0.31 g g<sup>-1</sup> by the parent strain. The  $\Delta gnd1$  mutant also showed increased ethanol yield to (0.38 g g<sup>-1</sup>). However, both mutants showed reduced rates of xylose uptake, which indicates that NADPH production is necessary for xylose assimilation in *S. cerevisiae*.

Xylitol production by native xylose-metabolizing yeasts varies a great deal with species and aeration conditions. *Pachysolen tannophilus*, *C. shehatae* and *Pichia stipitis* all produce xylitol to varying extents (Du Preez et al. 1984; Sanchez et al. 2002). *P. stipitis* is notable for its very low xylitol production and high ethanol yields. Ethanol production decreases and xylitol production increases in *P. stipitis* when the primary alcohol dehydrogenase is deleted (Cho and Jeffries 1998). This suggests that ADH competes with XDH for reductant—presumably NADH—in *P. stipitis*. In this organism, the main ADH is induced as oxygen availability decreases (Cho and Jeffries 1999; Passoth et al. 2003).

Lowering the cytosolic NADPH concentration while regenerating NAD<sup>+</sup> from NADH could reduce xylitol production (Richard et al. 2003). This might be accomplished by expressing a cytosolic transhydrogenase. *S. cerevisiae* does not possess transhydrogenase activity (Bruinenberg et al. 1985), so Nissen et al. (1997)

expressed a transhydrogenase gene from *Azotobacter vinlandii* in *S. cerevisiae* and then measured the intracellular concentrations of the NAD(P) and NAD(P)H cofactors. The concentrations of the nicotinamide cofactors in the glucose-grown control cells were as follows:

$$\begin{array}{ccccccc} \text{NADP}^+ & + & \text{NADH} & \longleftrightarrow & \text{NADPH} & + & \text{NAD}^+ \\ 0.23 & & 0.44 & & 1.21 & & 2.87 \\ & & & & & & \mu\text{mol g}^{-1} \\ & & & & & & \text{biomass} \end{array} \quad (1)$$

Expression of the transhydrogenase increased the production of 2-oxoglutarate and glycerol and shifted the intracellular ratio of (NADPH/NADP<sup>+</sup>):(NADH/NAD<sup>+</sup>) from 35 to 17 (Nissen et al. 1997). These results indicated that the thermodynamic equilibrium for the transhydrogenase reaction lies in the direction of NADH formation. So it seems unlikely that this approach will be useful in the absence of other energy-consuming reactions.

The XR encoded by *P. stipitis* *XYL1* has  $K_m=3.2$   $\mu\text{M}$  for NADPH and  $K_m=40$   $\mu\text{M}$  for NADH (Rizzi et al. 1988). The production of NADPH by glucose-6-phosphate dehydrogenase occurs largely on demand in response to the intracellular concentrations of NADPH (Michal 1999). Taken together, these three factors mean that the *P. stipitis* XR will always favor consumption of NADPH over NADH. Native *P. stipitis* does not produce significant amounts of xylitol, whereas recombinant *S. cerevisiae* expressing the *P. stipitis* *XYL1* produces abundant xylitol, so some factor other than XR must be responsible for enabling cofactor balance in *P. stipitis*.

### Respiration

Respiration plays a critical role in the metabolism of xylose by both native and engineered yeasts. The exact nature of the requirement is not fully understood, because oxygen appears to function differently in enabling growth and fermentation. Native xylose-metabolizing yeasts or genetically engineered *S. cerevisiae* (Eliasson et al. 2000b) can metabolize xylose to ethanol in the absence of oxygen, but oxygen is required for yeasts to grow on xylose (Ligthelm et al. 1988), and for optimal ethanol production, low aeration rates are required (Toivari et al. 2001). Anoxia kills *C. shehatae* when it is cultivated on xylose, but not when cultivated on glucose. This suggests that there is a fundamental difference in the oxygen requirements for the metabolism of these two sugars (Kastner et al. 1999).

Disruption of *CYC1* in *P. stipitis* blocks much of its capacity for ATP production and results in a petite colony morphology, even though this is a petite-negative yeast. *P. stipitis*  $\Delta cyc1$  mutants grow slowly on glucose or xylose, but unlike wild-type *P. stipitis*, they do not grow on glycerol or xylitol. Volumetric ethanol production rates of *P. stipitis*  $\Delta cyc1$  mutants on xylose are similar to the

parent, but because cell yields are much lower, the specific fermentation rate is about 50% higher with  $\Delta cyc1$  mutants (Shi et al. 1999). Loss of *CYC1* restricts cell growth but respiration capacity remains high because *P. stipitis* also possesses an alternative salicylhydroxamic acid-sensitive terminal oxidase, *STO1* (Shi et al. 2002). Disruption of *STO1* increases ethanol production from xylose but does not affect cell growth, which indicates that electron transfer to *Sto1* does not generate ATP.

## Anaerobiosis

One of the biggest challenges for commercialization of the yeast xylose fermentation is obtaining growth on xylose under anaerobic conditions. The cost of aerating bioreactors for ethanol production is prohibitive by current practice. As was first noted by Wang and Schneider in 1980, *S. cerevisiae* and other yeasts can grow on xylulose under aerobic conditions (Wang and Schneider 1980), but even though they can ferment xylulose, they do not grow on this sugar under anaerobic conditions (Maleszka and Schneider 1984). Mitochondrial function appears to be necessary for the growth of yeasts on xylose or xylulose. Ethanol production from xylose, xylitol or xylulose is enhanced by aeration in most yeasts (Maleszka and Schneider 1982); and in some species, such as *C. tropicalis*, aeration is essential for ethanol production (Jeffries 1981).

Eliasson et al. (2000b) were the first to report the anaerobic production of ethanol from xylose by recombinant *S. cerevisiae* TMB3001. However, this yeast grew on xylose only in the presence of oxygen; and glucose was included in all fermentations to enable continuous cultivation. Xylose was co-utilized with glucose under anaerobic conditions. Xylose uptake varied inversely with its concentration, but even at the highest xylose concentration, (15 g xylose l<sup>-1</sup>, 5 g glucose l<sup>-1</sup>) only 12% of the xylose was consumed. The anaerobic ethanol yield on xylose was estimated at 0.21 g g<sup>-1</sup>, assuming a constant ethanol yield on glucose. Glucose appears to be required for the anaerobic metabolism of xylose, because these authors were not able to maintain a steady state when cultures were grown on xylose alone (Eliasson et al. 2000b).

To overcome this limitation, Sonderegger and Sauer (2003) maintained *S. cerevisiae* TMB3001 in continuous culture under progressively more restrictive oxygen limitations. Starting from continuous aerobic cultivation on a mixture of xylose and glucose and progressing to anaerobic cultivation on xylose alone, the authors obtained two cell populations. Clones taken from the larger population grew anaerobically on xylose but showed impaired growth on glucose. Clones taken from the smaller population were incapable of anaerobic growth but produced more ethanol from xylose than the parental strain.

In a separate research effort, Wahlbom et al. (2003) also used continuous cultivation under aerobic, oxygen-limited

and anaerobic conditions to obtain improved mutants of recombinant *S. cerevisiae* with higher capacities for xylose fermentation. However, even the best of these mutants showed only about one-third of the aerobic maximum growth rate and two-thirds of the ethanol productivity obtained with *P. stipitis* CBS 6054 on xylose (Wahlbom et al. 2003).

*C. shehatae* can use glucose and xylose simultaneously in a chemostat, but aeration is required for cell growth (Kastner et al. 1998). *C. shehatae* cells, cultivated aerobically on D-glucose and D-xylose, undergo one doubling or less following a shift to anoxia. Cell viability declines nine times faster in D-xylose than in D-glucose fermentations. Anaerobic growth does not occur on either D-glucose or D-xylose (Kastner et al. 1999).

Heterologous expression of *S. cerevisiae* *URA1* in *P. stipitis* was reported to confer anaerobic growth, but this apparently required an uncharacterized mutational event in the host cell background, because transformed cells required more than 100 h before initial growth was noted (Shi and Jeffries 1998).

For commercial purposes, it may be possible to cultivate either native or engineered yeasts anaerobically on glucose followed by a respiro-fermentative phase on the residual xylose. Glucose and xylose are almost always obtained as mixtures from lignocellulose hydrolysates, so by properly engineering cell recycle loops, it should be possible to obtain high-yield conversions under anaerobic or oxygen-limited conditions.

## Metabolite flux and transcriptome profiling

Metabolic flux analysis and flux estimates based on <sup>13</sup>C-labeling experiments (Christensen et al. 2002) have been used to estimate metabolite levels of *S. cerevisiae* and *P. stipitis* grown on glucose and xylose. Intracellular metabolite levels are higher in industrial yeast strains than in laboratory strains engineered for xylose utilization (Zaldivar et al. 2002). *P. stipitis* derives at least 58% of its phospho-enol-pyruvate (PEP) through the non-oxidative PPP, whereas *S. cerevisiae* uses the non-oxidative PPP for the biosynthesis of less than 4% of its PEP (Fiaux et al. 2003). A flux balance analysis (FBA) showed that the maximum ethanol yield from xylose in yeast is 0.46 g g<sup>-1</sup> rather than 0.51 g g<sup>-1</sup>, because of the cofactor difference between XR and XDH. Metabolic FBA also predicted that there is an optimal aeration level for ethanol production and that xylitol accumulation decreases with higher aeration. Both of these predictions have been confirmed by measuring product yields at various aeration rates (Jin 2002).

Transcriptome profiling methods, such as RT-PCR and microarray experiments, were applied to monitoring the differential expression of genes between glucose and xylose fermentation by recombinant *S. cerevisiae*. Of the 5,944 genes detected under oxygen-limited culture conditions, 386 (6.6%) showed differential expression when cells were grown on xylose, as compared with glucose. As

expected, most of those genes fell in the energy-production category. Expression levels of genes coding for glycolytic, fermentative and pentose phosphate enzymes did not change greatly. However, expression of the genes encoding tricarboxylic acid cycle and respiratory enzymes greatly increased when cells were grown on xylose (Jin 2002).

### Protoplast fusion

Protoplast fusion is widely used to improve the fermentative properties of industrial yeasts. By complementing multiple auxotrophic markers, it is possible to obtain stable hybrids between closely related species. In an attempt to increase yeast strains with ethanol tolerance and the ability to ferment xylose, *S. cerevisiae* has been fused with auxotrophic strains of *C. shehatae* or *P. stipitis* (Gupthar 1992), but mononucleate fusants quickly segregate into their parental type strains (Yoon et al. 1996). Other researchers report fusants between *S. cerevisiae* and *P. stipitis* that show the capacity for xylose fermentation and an ability to ferment glucose in the presence of 6% ethanol (Kordowska-Wiater and Targonski 2001). Given the instability of the hybrids, this will probably not lead to commercial yeast strains.

### Xylitol production

Native xylose-metabolizing yeasts, such as *Pachysolen tannophilus*, *C. shehatae*, (Du Preez et al. 1984), *C. boidinii* (Vandeska et al. 1996; Winkelhausen et al. 1996), *Hansenula polymorpha* (Sanchez et al. 1998) and *C. guilliermondii* (Rodrigues et al. 2002), all produce xylitol

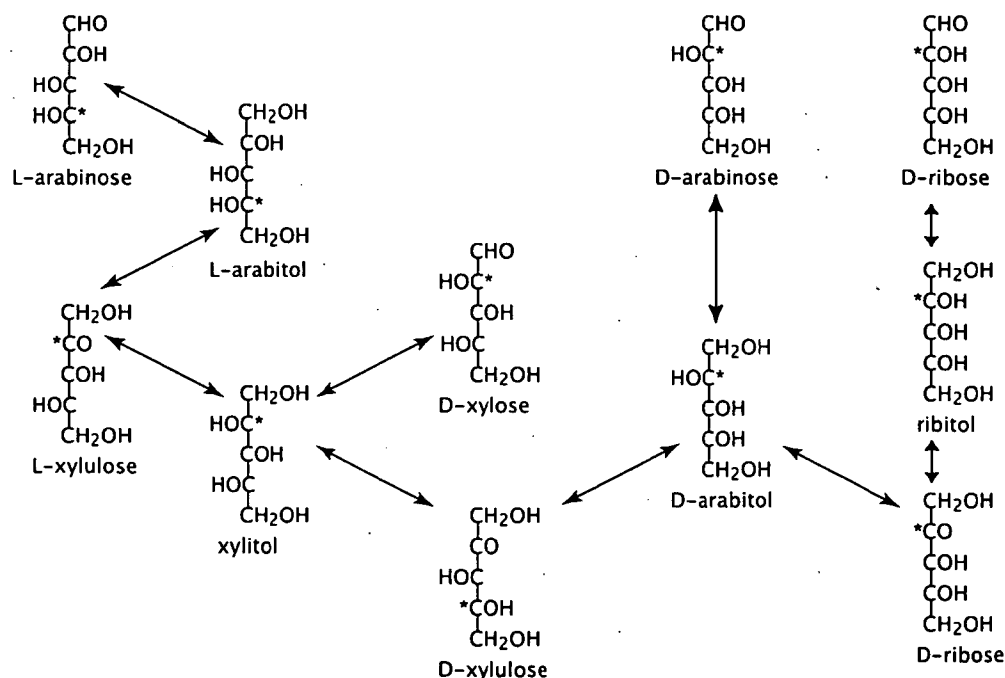
to greater or lesser degrees depending on the pH, oxygen availability and other culture conditions. *Pichia stipitis* is notable because it produces relatively little xylitol (Sanchez et al. 2002). When its genes for Adh are disrupted, xylitol production increases dramatically (Cho and Jeffries 1998); and when the D-xylulokinase gene is disrupted, *P. stipitis* produces a mixture of xylitol and arabitol (Jin et al. 2002). *S. cerevisiae* cells engineered for xylose utilization tend to produce xylitol (Kötter and Ciriacy 1993; Hallborn et al. 1994; Tantirungkij et al. 1994; Walfridsson et al. 1995; Eliasson et al. 2000b). If many copies of *XYL1* are integrated into the genome, *S. cerevisiae* transformants are stable and can produce xylitol in sequential batch or continuous culture (Kim et al. 1999). By increasing the expression of *XYL2* relative to *XYL1*, it is possible to decrease xylitol secretion (Jin et al. 2003).

### Arabinose utilization

The utilization of L-arabinose is particularly important in the conversion of corn hulls to ethanol (Saha et al. 1998). Corn fiber consists of about 20% starch, 14% cellulose and 35% hemicellulose; and L-arabinose makes up approximately 28% of the hemicellulosic fraction (Park et al. 2001). An extensive screen of 116 yeasts that can grow on L-arabinose showed that four strains, *C. aurangiensis*, *C. succiphila*, *Ambrosiozyma monospora* and *Candida* sp. (YB-2248) could produce some ethanol (4.1 g l<sup>-1</sup> or less) directly from arabinose (Dien et al. 1996). While the production rates were very low, these studies showed for the first time that yeasts can directly convert L-arabinose to ethanol (Fig. 2).

The pathways for L-arabinose and D-arabinose metabolism are distinct in yeasts. *P. stipitis* will grow very

Fig. 2 Interconversion of pentoses and pentitols by NAD<sup>+</sup> (PH)-mediated oxidoreductases



slowly on L-arabinose, but it does not ferment this sugar. A mutant of *P. stipitis* that was unable to metabolize L-arabinose could grow on D-arabinose (Shi et al. 2000). Complementation of this mutant with *XYL2* restored growth on L-arabinose. This showed that the pathway used by *P. stipitis* for L-arabinose metabolism is similar to that used by *Aspergillus niger* (Witteveen et al. 1989).

Relatively little is known about L-arabinose uptake by yeasts. In *C. shehatae*, it appears to be mediated by proton symport (Lucas and van Uden 1986). L-Arabinose is similar in structure to D-galactose (Rees 1977) and its transport in *S. cerevisiae* is mediated by *GAL2* (Kou et al. 1970). In *Kluyveromyces lactis*, the *LAC12* gene codes for an inducible lactose permease that is similar in structure to the *Escherichia coli* xylose- $H^+$  and arabinose- $H^+$  transporters (Chang and Dickson 1988). D-Arabinitol dehydrogenase (Hallborn et al. 1995) functions in an alternative pathway that connects D-xylulose to D-ribulose (Jin et al. 2002).

Fungi metabolize L-arabinose through five enzymes, aldose (xylose) reductase, L-arabinitol 4-dehydrogenase (*lad1*; Richard et al. 2001), L-xylulose reductase (*lxr1*; Richard et al. 2002), xylitol dehydrogenase and D-xylulokinase. Overexpression of *lad1* and *lxr1* along with *XYL1*, *XYL2* and *XKS1* enabled *S. cerevisiae* to grow on and ferment L-arabinose (Richard et al. 2003). Ethanol production occurred at a very low rate. About 0.1 g of ethanol was formed by 4 g of cells in 70 h under anaerobic conditions. Under aerobic conditions, the ethanol formed from L-arabinose would probably be re-assimilated.

In a different approach to engineer *S. cerevisiae* for L-arabinose fermentation, Sedlak and Ho (2001) expressed three genes of the *araBAD* operon from *E. coli* in *S. cerevisiae*. They reported activity with all three enzymes, but the transformant did not produce ethanol from L-arabinose (Sedlak and Ho 2001). Becker and Boles (2003) were more successful in this approach. Unlike Sedlak and Ho, they were not able to obtain activity through the heterologous expression of *E. coli* *araA*, which codes for L-arabinose isomerase, but they were able to express the *araA* gene from *Bacillus subtilis* and this—along with the heterologous expression of *E. coli* *araB* and *araD* plus overexpression of *S. cerevisiae* *GAL2*—gave rise to a yeast strain that could grow slowly on L-arabinose. After more than 200 h of cultivation, a transformant arose that could grow on L-arabinose, with a doubling time of about 8 h. They were able to identify two mutational events—one in the bacterial L-ribulokinase that reduced affinity for L-ribulose and one in the yeast genome that increased transaldolase expression. Together, these enabled growth on and fermentation of L-arabinose. The resulting strain produced up to 0.08 g ethanol g<sup>-1</sup> biomass h<sup>-1</sup>. This represents a major breakthrough in the metabolic engineering of arabinose metabolism in yeast.

## Cellulase and xylanase expression

Native strains of *P. stipitis* produce xylanases (Ozcan et al. 1991) that enable the fermentation of xylan directly to ethanol, but the yields are very low (Lee et al. 1986). By increasing xylanase production either through mutation (Basaran et al. 2000) or heterologous expression (Morosoli et al. 1993; Den Haan and Van Zyl 2001), it is possible to enhance the xylan fermentation rate. Yeast  $\beta$ -xylosidase is probably most important for the fermentation of xylobiose and xylotriose, because these are the most conspicuous products of endoxylanase xylanase activity and they are also formed during the acid hydrolysis of xylan. Several researchers have heterologously expressed xylanases in *S. cerevisiae* (Den Haan and Van Zyl 2001; La Grange et al. 2001).

## Spent sulfite and hydrolysate fermentation

In the final analysis, the ability of an organism to ferment sugars in hemicellulosic hydrolysates determines the success of metabolic engineering efforts. Acetic acid and toxic phenolic products from lignocellulose can inhibit growth of yeasts in hydrolysates. However, post-hydrolysis treatments can reduce toxicity and strains can be selected for resistance. The most cost-effective hydrolysate treatment—calcium over-liming—is also one of the oldest. Over-liming is used to prepare sulfite waste liquors (Nigam 2001b) and acid hydrolysates of hardwood (Nigam 2001a). Unfortunately, acid hydrolysis and over-liming produce large amounts of calcium sulfate that must be removed. The toxicity of hydrolysates can be significantly reduced by adding laccase to polymerize the free phenolic compounds (Jönsson et al. 1998). A laccase cloned from *Trametes versicolor* has been expressed at a high level in *S. cerevisiae* (Larsson et al. 2001). *S. cerevisiae* TMB 3001 grew better in laccase-treated hydrolysate than in untreated hydrolysate, but it showed almost no utilization of xylose. In a comparison of *P. stipitis* with *C. shehatae*, the latter yeast was better able to ferment hydrolysates (Sreenath and Jeffries 2000). In simultaneous saccharification and fermentation, yields of 0.47 g g<sup>-1</sup> substrate were obtained (Sreenath et al. 1999, 2001). Wild-type strains of *C. shehatae* can ferment rice straw autohydrolysates to ethanol with yields of 0.45 g ethanol g<sup>-1</sup> sugar consumed and they can produce 0.37 g g<sup>-1</sup> from acid prehydrolysates (Abbi et al. 1996). The ethanol productivity of *P. stipitis* in wood hydrolysates can be improved by up to 2-fold by selecting for resistant strains (Nigam 2001a).

## Prospects for future progress

Metabolic engineering enables *S. cerevisiae* to ferment D-xylose and L-arabinose to ethanol and it improves the capacity of native xylose-fermenting yeasts, such as *P. stipitis*. The improvement obtained with any one change

has been incremental. Co-production of xylitol, low ethanol production rates, requirements for oxygen and co-metabolizable carbon sources remain problems with recombinant *S. cerevisiae*. Most trials use haploid laboratory strains rather than industrial yeasts for their genetic backgrounds and trials with mixed sugar hydrolysates are not often reported. Mutagenesis and strain selection has improved xylose utilization in recombinant strains, but most mutations have not been characterized. In some instances, multiple genes have been altered through deletion or overexpression, but rarely have expression levels been manipulated. There are, therefore, many opportunities to obtain further improvements by learning more about the factors that limit xylose utilization under anaerobic conditions, by selecting better genetic backgrounds for heterologous expression, by expressing multiple genes at optimal levels and by combining the various beneficial traits into single strains. While strain improvement will probably continue for several years, ethanol production rates and yields are becoming practicable for some commercial applications.

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**Recent Advances in Petroleum Microbiology**

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## INTRODUCTION

Petroleum is a complex mixture of hydrocarbons and other organic compounds, including some organometallo constituents, most notably complexing vanadium and nickel. Petroleum recovered from different reservoirs varies widely in compositional and physical properties. Long recognized as substrates supporting microbial growth (92, 580), these hydrocarbons are both a target and a product of microbial metabolism (169). Biodegradation by microorganisms modifies waxy crude oils in beneficial ways, but conditions for down-hole applications require the use of thermophiles, resistant to organic solvents, with heat-stable enzymes and reduced oxygen requirements (21, 48).

A wide range of studies have dealt with biotransformation, biodegradation, and bioremediation of petroleum hydrocarbons (30, 31, 48, 415, 490, 523), and interest in exploiting

petroleum-degrading organisms for environmental clean-up has become central to petroleum microbiology (29). A common theme of early reviews focused on the examination of factors, including nutrients, physical state of the oil, oxygen, temperature, salinity, and pressure, influencing petroleum biodegradation rates, with a view to developing environmental applications (29). Metabolic studies were implemented on the aerobic pathways for alkane, cycloalkane, and aromatic and polycyclic aromatic hydrocarbon (PAH) biodegradation (103, 104, 294, 301, 479, 572, 596, 656), for transformations of nitrogen and sulfur compounds (55, 74, 75, 299, 352, 417), and, more recently, the microbial mechanisms of anaerobic hydrocarbon catabolism (203, 243, 250, 581, 390, 482, 664).

Most significantly, through the developments and applications of molecular techniques, our understanding of the processes of hydrocarbon catabolism has advanced substantially, and many novel catalytic mechanisms have been characterized. A molecular approach is also contributing to a more detailed characterization of bacterial membrane structure. We are learning a great deal about cellular and other physiological adaptations to the presence of hydrocarbons, as well as the

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biochemical mechanisms involved in hydrocarbon accession and uptake (143, 251, 566). The use of genetically engineered microbes for bioremediation has also been considered (210).

The vast range of substrates and metabolites present in hydrocarbon-impacted soils surely provides an environment for the development of a quite complex microbial community. Culture-based methods and culture-independent methods are being developed and implemented to improve our understanding of these microbial communities. Isolating and identifying microorganisms responsible for hydrocarbon transformations have long been recognized as important from a fundamental and applied viewpoint, and lists of hydrocarbon-degrading organisms (bacteria, yeasts, fungi, and algae) are available (30, 33, 366, 522). Leahy and Colwell (366) discussed colony hybridization and dot blot assays in their review and cited molecular tools as revolutionary for describing microbial communities. Magot et al. (398) recently reviewed the current state of knowledge of microorganisms from petroleum reservoirs, including mesophilic and thermophilic sulfate-reducing bacteria, methanogens, mesophilic and thermophilic fermentative bacteria, and iron-reducing bacteria. Again, molecular tools were called upon to provide more detailed community characterizations. These and related studies should provide us with new information on the long-term ecological effects of petroleum pollution and give us directions, for example, regarding the development of new remedial approaches and methods to control some of the deleterious microbial activities occurring during petroleum production.

Current applied research on petroleum microbiology encompasses oil spill remediation (490, 492, 598), fermentor- and wetland-based hydrocarbon treatment (212, 281, 336, 530, 569), biofiltration of volatile hydrocarbons (176), microbial enhanced oil recovery (42, 153), oil and fuel upgrading through desulfurization (417, 554) and denitrogenation (55), coal processing (102), fine-chemical production (412, 415), and microbial community-based site assessment (394). The roles and practical applications of chemical and biological surfactants have been widely reviewed (260, 454, 529, 643).

Oil spill treatment on shorelines and problems associated with open-ocean remediation have been discussed through case histories in numerous reviews (30, 31, 44, 489, 599). Other practical applications include land- and reactor-based refinery waste treatment, in situ tanker ballast cleaning, and subsurface remediation (31, 44).

Heavy crude oil recovery, facilitated by microorganisms, was suggested in the 1920s and received growing interest in the 1980s as microbial enhanced oil recovery (153). As of 1998, only one productive microbial enhanced oil recovery project was being carried out in the United States (613), although in situ biosurfactant and biopolymer applications continue to garner interest (42).

A limited number of studies have been carried out on biological methods of removing heavy metals such as nickel and vanadium from petroleum distillate fractions, coal-derived liquid shale, bitumens, tars, and synthetic fuels (188, 429, 487, 488, 673). In one approach, cytochrome *c* reductase and chloroperoxidase enzymes have shown potential for metal removal from petroleum fractions. However, further characterization on the biochemical mechanisms and bioprocessing issues in-

involved in heavy metal removal are required in order to develop a reliable biological process.

Bacteria with selected petroleum-metabolizing enzymes amenable to being linked to electronic interfaces are being engineered and developed as biosensors (142). These systems have applications in monitoring environmental contaminant concentrations and toxicities during implementation of remedial processes and also have potential applications in control of environmental processes.

This review deals with developments in our knowledge of petroleum microbiology and in the application of microorganisms in oil bioprocesses and as biosensors. Advances in our understanding of microbial catabolism are presented, including an evaluation of the biochemical mechanisms that control microbial responses to hydrocarbon substrates. These aspects include changes in membrane architecture, active uptake and efflux of hydrocarbons and chemotaxis, and the potential for coordinate control of some of these systems to allow metabolism to take place. Developments in oil bioprocessing focus on transformation of wastes and on the production and upgrading of petroleum and petrochemicals, with emphasis placed on maximizing the rates and extents of microbial growth, hydrocarbon accession, and transformation. Sections dealing with desulfurization and fine-chemical synthesis additionally illustrate the potential benefits of recombinant strains containing enzymes with enhanced activity and/or altered substrate specificity. The possible use of biosensors for online monitoring of pollutants is also addressed.

## METABOLISM

### Aerobic Alkane Metabolism

Microorganisms are equipped with metabolic machinery to use petroleum as a carbon and energy source. The fundamental aspects of *n*-alkane metabolism and the genes involved have been known for some time. While significant gains have been made in our understanding of the processes involved, the specifics of individual systems and the diversity of systems are yet to be fully described. This section will highlight the recently discovered variability in both the regulation and clustering of alkane degradation genes between species as well as the realization that a single strain may carry multiple genes that code for different enzymes carrying out similar functions. A few rare metabolic pathways will also be discussed.

From a regulatory genetic standpoint, the most extensively characterized alkane degradation pathway is encoded by the OCT plasmid carried by *Pseudomonas putida* Gpo1 (formerly *Pseudomonas oleovorans*) (626, 627). Here, a membrane-bound monooxygenase and soluble rubredoxin and rubredoxin reductase serve to shunt electrons through NADH to the hydroxylase for conversion of an alkane into an alcohol. The alcohol can be further oxidized to an aldehyde and acid prior to proceeding into the  $\beta$ -oxidation and tricarboxylic acid cycles. Recently, van Beilen et al. (626, 627) studied the OCT plasmid, while Canosa et al. (98) and Panake et al. (470) examined expression of the AlkS regulator, and Yuste et al. (683, 684) studied the catabolite repression system.

A model for alkane metabolism, including the locations of the Alk proteins and regulation of the *alk* genes, is shown in

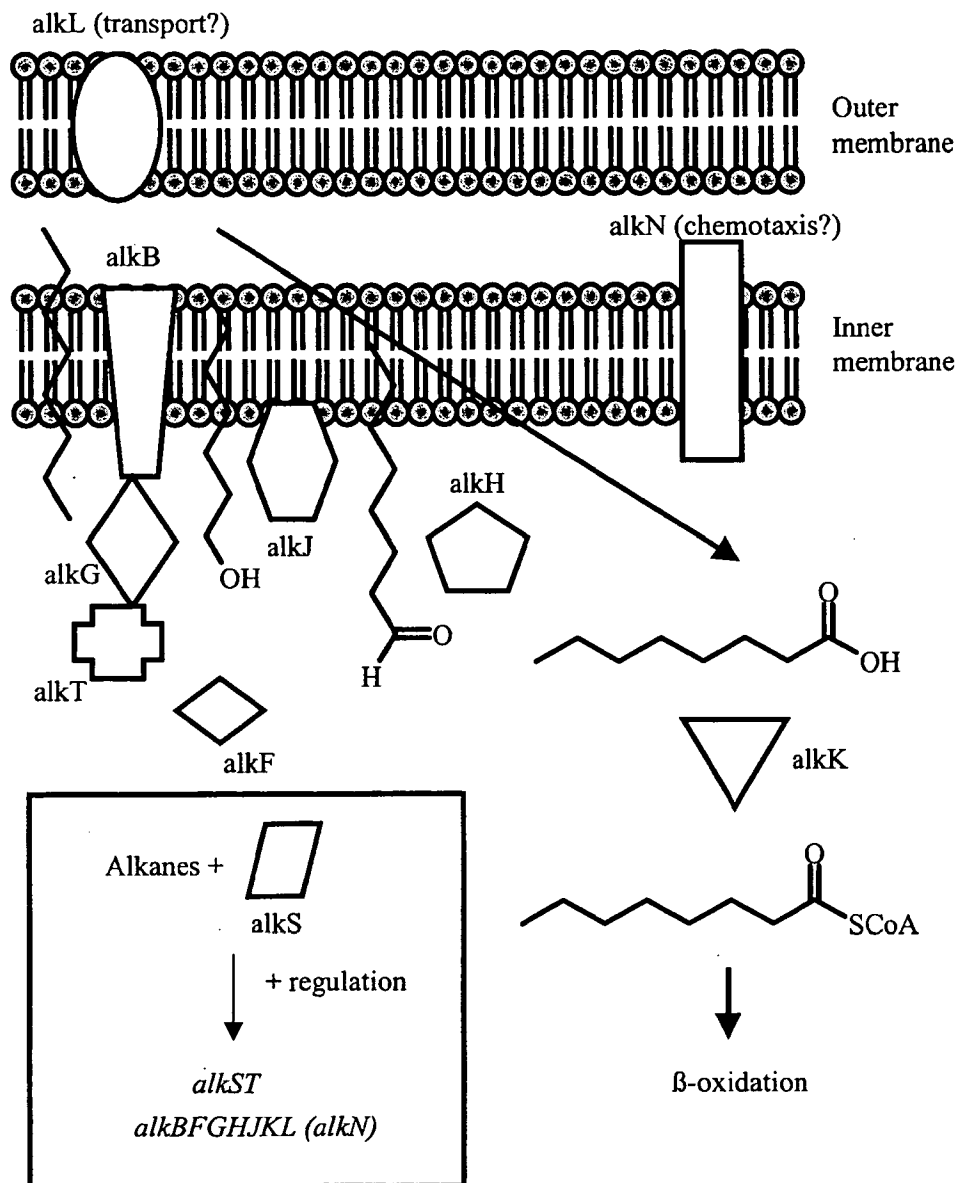


FIG. 1. Schematic of alkane degradation in gram-negative bacteria, showing the locations and functions of the *alk* gene products. The products include AlkB (alkane hydroxylase), AlkF and AlkG (rubredoxins), AlkH (aldehyde dehydrogenase), AlkJ (alcohol dehydrogenase), AlkK (acyl-CoA synthetase), AlkL (outer membrane protein that may be involved in uptake), AlkN (a methyl-accepting transducer protein that may be involved in chemotaxis), AlkT (rubredoxin reductase), and AlkS (positive regulator of the *alkBFGHIJKL* operon and *alkST* genes).

Fig. 1 (627). Here, the *alkBFGHIJKL* operon encodes the enzymes necessary for converting alkanes into acetyl-coenzyme A (CoA), while *alkST* encode a rubredoxin reductase (AlkT) and the positive regulator for the *alkBFGHIJKL* operon (AlkS). These two operons are located end to end, separated by 9.7 kb of DNA, within which lies *alkN*, a gene coding for a methyl-accepting transducer protein that may be involved in alkane chemotaxis. Note that of all the genes described, the function of *alkL* remains unknown, although it is suspected to be involved in transport. Comparative analysis of insertion sequences in *P. putida* P1 and the previous observation that the G+C content of the *alk* genes is lower than that of both the host strain and the OCT plasmid suggest that the genes are

part of an integrated mobile element. Two other plasmid systems have been partially characterized: the OCT plasmid in *Pseudomonas maltophilia* has an *alkA* gene distinct from that of *P. putida* (374), and the unique pDEC plasmid in *Pseudomonas* sp. strain C12B (347).

As other strains are characterized, it appears that the clustering and regulation of alkane degradation genes varies among the bacteria. *Burkholderia cepacia* has an *alkB* gene that is not linked to other alkane degradation genes as it is in *P. putida* (408). The *PalkB* promoter in this organism is down-regulated by catabolite repression more strongly than in *P. putida* GPO1 (683). Other differences include the repression of alkane degradation by citrate and the maintenance of repres-

sion during stationary phase in *B. cepacia*, two phenomena not observed in *P. putida* GPO1. In *Acinetobacter* sp. strain ADP1, *alkM*, the terminal alkane hydroxylase-encoding gene, is regulated by *alkR*, which shows no similarity to the LuxR-UhpA-like *alkS* regulator in *P. putida*. In addition, the genes in *Acinetobacter* sp. strain ADP1 are not found in a large operon or on a plasmid. Indeed, the genes are 396 kb from *rubA* and *rubB*, which encode rubredoxin and rubredoxin reductase (213, 505).

The *alkM*, *rubA*, and *rubB* genes in *Acinetobacter* sp. strain M1 are homologous to those in *Acinetobacter* sp. strain ADP1. Interestingly, two alkane hydroxylase complexes (*alkMa* and *alkMb*) whose expression is controlled by *n*-alkane chain length are present in this strain. Conversely, the rubredoxin and rubredoxin reductase are constitutively expressed. Hydrophathy plots of *AlkMa* and *AlkMb* suggest that the proteins are similar to *AlkB* in *P. putida* in that they are membrane bound. *AlkMa* appears to be similar to *AlkM* of *Acinetobacter* sp. strain ADP1. The first of two transcriptional regulators in *Acinetobacter* sp. strain M1 (*AlkRa*) is related to AraC-XylS type regulators, which includes that of *Acinetobacter* sp. strain ADP1. The second regulator (*AlkRb*) is similar to *OruR* of *P. aeruginosa*. The two regulators are induced by different *n*-alkanes in this strain. *alkMa* responds to solid, long-chain alkanes ( $>C_{22}$ ), while *alkMb* responds to liquid alkanes ( $C_{16}$  to  $C_{22}$ ). Unlike the case in *P. putida*, neither acetate nor hexadecanol induces *alkMa* and *alkMb* (602).

The presence of multiple alkane hydroxylase genes in a single strain does not appear to be a unique phenomenon. Two distinct monooxygenases, a Cu-containing monooxygenase and an integral-membrane, binuclear-iron monooxygenase similar to that of *P. putida* GMO1 have been described in *Nocardioides* sp. strain CF8 (233). While the Cu-containing monooxygenase is expressed in response to a wide range of alkanes, only those with more than six carbons induce the binuclear-iron monooxygenase. Once again, the genes encoding alkane metabolism in *Acinetobacter* sp. strain M1 and *Nocardioides* sp. strain CF8 are not clustered together as in the OCT plasmid (275, 602). Other enzymes involved in *Acinetobacter* sp. strain M1 alkane metabolism have been characterized. Ishige et al. (275) isolated a soluble long-chain  $NAD^+$ -dependent aldehyde dehydrogenase whose activity increased with increasing aldehyde chain length (tetradecanal preferred) that is encoded by the chromosomal *ald1* gene. This enzyme plays a role in both alkane degradation and biosynthesis, depending on the conditions. The  $NAD^+$ -dependent aldehyde dehydrogenase in strain HD1 is also reported to prefer long-chain aldehydes (462). A thermostable  $NADP^+$ -dependent medium-chain alcohol dehydrogenase, encoded by *alrA*, has also been isolated but is not believed to participate in the main alkane oxidation pathway due to its cytosolic location and greater activity towards medium-chain alcohols (603).

Despite the importance of alkane degradation systems, little information is available for pathways other than the aerobic monooxygenase-mediated pathway found on the OCT plasmid. Evidence for the Finnerty pathway, where a dioxygenase converts alkanes to aldehydes through *n*-alkyl hydroperoxides without an alcohol intermediate, has been described for *Acinetobacter* sp. strain M1 (397, 534). The dioxygenase requires molecular oxygen to catalyze the oxidation of *n*-alkanes ( $C_{10}$  to  $C_{30}$ ) and alkenes ( $C_{12}$  to  $C_{20}$ ) without the production of oxygen

radicals. A flavin adenine dinucleotide chromophore was detected, and the enzyme is thought to contain  $Cu^{2+}$ . Unlike the case for the 1-monooxygenase in *P. putida*, rubredoxin and  $NAD(P)H$  are not required.

Another novel metabolic pathway has been observed in a *Rhodococcus* mutant (338). In this case, aliphatics are *cis*-desaturated, producing products with double bonds mainly at the ninth carbon from the terminal methyl group. It is postulated that a coenzyme A-independent *cis*-desaturase may be involved in this activity. Dutta and Harayama (159) recently noted that the degradation of the long side chains of *n*-alkylbenzenes and *n*-alkylcyclohexanes by *Alcanivorax* sp. strain MBIC 4326 proceeds mainly by  $\beta$ -oxidation (Fig. 2). However, minor products suggest the possibility of other degradative routes. For example, 4-cyclohexylbutanoic acid was metabolized through 4-cyclohexyl-2-butenic acid ( $\beta$ -oxidation) and other intermediates not believed to be formed by  $\beta$ -oxidation (4-cyclohexyl-3-butenic acid and cyclohexylcarboxylic acid).

In the above cases, there is much work to be done with respect to describing both the genetic systems and the enzymes involved. Even more challenging will be answering questions such as what role these pathways play in environmental remediation, how the different approaches to alkane metabolism evolve and how are they related, and how well-characterized and novel metabolic pathways can be applied in fine-chemical synthesis.

### Aerobic PAH Metabolism

A great deal of work has been carried out in trying to rationalize the persistence of PAH in the environment. As more studies are carried out, it is becoming increasingly evident that a vast array of microbial species (bacteria, fungi, algae, and cyanobacteria) have a diversity of tools to use both low- (three rings or fewer) and high-molecular-weight (four or more rings) PAHs such as naphthalene, acenaphthene, anthracene, fluoranthene, pyrene, and chrysene as sole carbon and energy sources. While no strains have yet been found to utilize PAHs with more than four rings, such as benzo[a]pyrene as a sole carbon and energy source, cometabolic transformations have been characterized (for reviews, see references 103, 104, 294 301, 572, 596, and 597).

The low water solubility and high sorption capacity of PAHs are often found to greatly influence biodegradation, but other factors, including production of toxic or dead-end metabolites, metabolite repression, the presence of preferred substrates, and the lack of cometabolic or inducer substrates, must be considered when PAH persistence is evident (433, 295). Understanding how these factors affect the transformation of and determining any given PAH is difficult; understanding the processes in natural environments when mixtures of PAHs and their myriad metabolites are present is more difficult, especially as the majority of work has focused on a narrow selection of species. Indeed, the cited reviews generally conclude by calling for more study into the regulation of PAH biodegradation, biodegradation of PAH mixtures, and interactions within microbial consortia.

Until recently, the majority of information on the genetics of PAH metabolism has come from studying naphthalene catabolic plasmids such as NAH7 from *Pseudomonas putida* strain

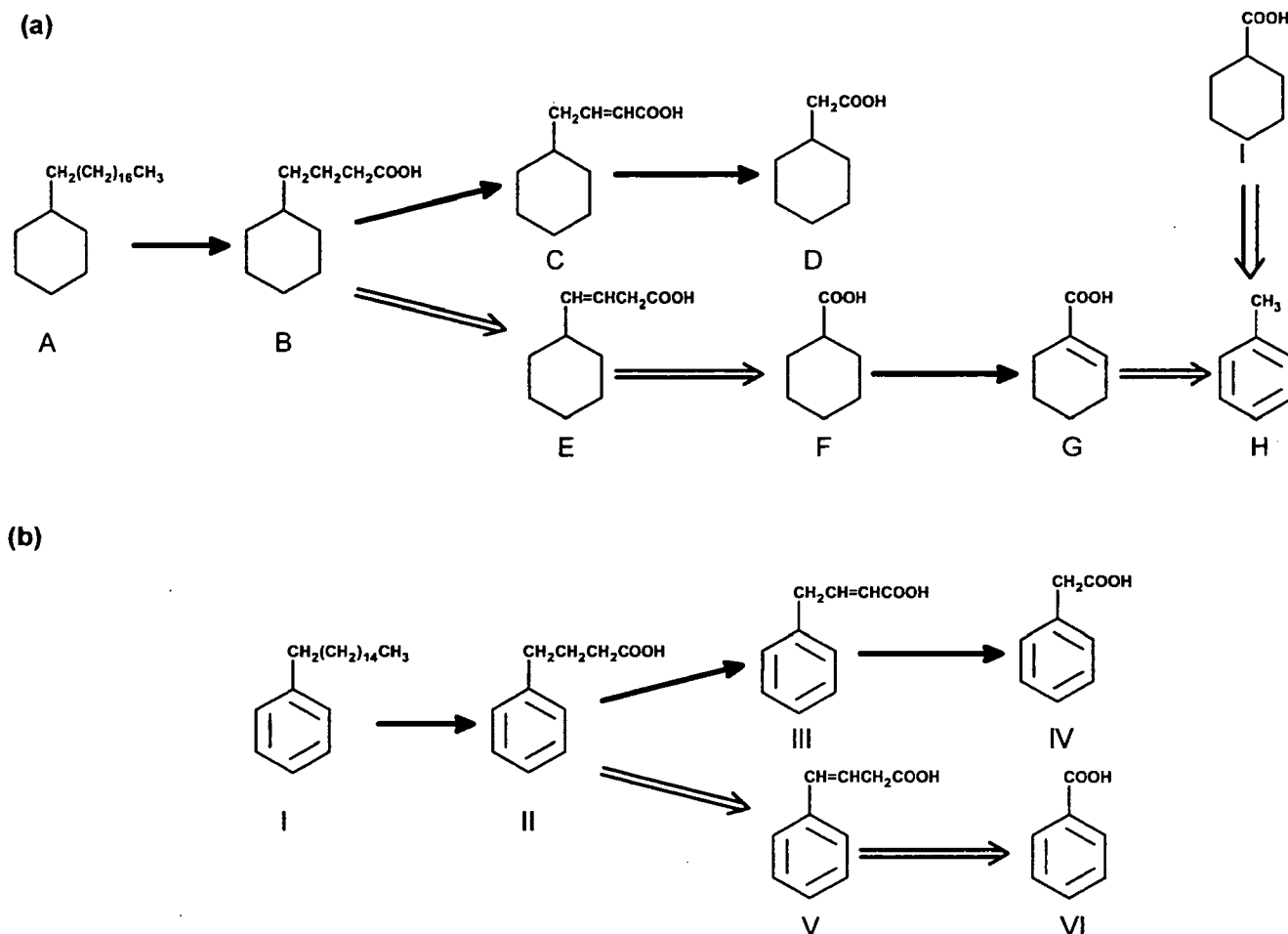


FIG. 2. Proposed metabolic pathway illustrating biodegradation of an *n*-alkylcyclohexane (a) and an *n*-alkylbenzene (b) by an *Alcanivorax* sp. strain MBIC4326 (adapted from reference 159). The major metabolic route of  $\beta$ -oxidation is shown with bold arrows, while minor routes are indicated with open arrows and a novel metabolic route by large open arrows. Pathway a: A, *n*-octadecylcyclohexane; B, 4-cyclohexabutanoic acid; C, 4-cyclohexyl-2-butanoic acid; D, cyclohexane acetic acid; E, 4-cyclohexyl-2-butenic acid; F, cyclohexane carboxylic acid; G, 1-cyclohexene-1-carboxylic acid; H, benzoic acid; I, 3-cyclohexene-1-carboxylic acid. Pathway b: I, *n*-hexadecylbenzene; II, 4-phenylbutanoic acid; III, 4-phenylbutenoic acid; IV, phenylacetic acid; V, 4-phenylbutenoic acid; VI, benzoic acid.

G7. In this well-characterized system, the first operon (*nahAaAbAcAdBFCEd*) encodes the pathway for naphthalene conversion to salicylate (upper pathway), and the second (*nahGTHINLOMKJ*) codes for the conversion of salicylate via catechol *meta*-cleavage to acetaldehyde and pyruvate (lower pathway) (164, 485, 568, 679). The regulator for both operons is encoded by a third operon containing *nahR*, which is induced by salicylate (547). Here, molecular oxygen is introduced into the aromatic nucleus via naphthalene dioxygenase, a multi-component nonheme iron oxygenase enzyme system consisting of a reductase, a putative Rieske [2Fe-2S] iron sulfur center in a ferredoxin, and an iron-sulfur flavoprotein. The initial reaction results in the formation of *cis*-naphthalene dihydrodiol, which is subsequently converted to salicylate and then to tri-carboxylic acid intermediates (for more detail, see references 104, 220, and 679). As will be discussed below, naphthalene dioxygenase is now known to be a versatile enzyme, able to catalyze a wide variety of reactions. Molecular and biochemical evidence that the naphthalene plasmid degradative enzyme system could mineralize other PAHs, such as phenanthrene

and anthracene, was first provided by two research groups in 1993 (423, 540).

As more PAH-degrading bacteria were isolated and characterized, and as molecular methods to study microbial communities developed, the diversity of PAH metabolic genes was discovered. Examples of bacteria with unknown, nonhomologous genes to the naphthalene NAH7-like catabolic plasmids have been reported recently (318, 528). At the same time, a variety of new isofunctional gene sequences have been reported in different bacterial species, most notably in *Nocardia*, *Rhodococcus*, and *Mycobacterium* spp., some of which are capable of using high-molecular-weight PAHs such as pyrene as carbon and energy sources (Table 1).

High levels ( $\approx 90\%$ ) of homology and a conserved gene arrangement are observed in the *nah*, *ndo*, *pah*, and *dox* sequences (63, 64, 147, 333, 355, 601). In fact, it has been proposed that the *dox* plasmid, which encodes a dibenzothiophene (DBT) metabolic pathway analogous to the naphthalene catabolic pathway, may in fact be a naphthalene catabolic plasmid (163). High homology, however, does not necessarily translate



TABLE 1. Chromosomally and plasmid-encoded polycyclic aromatic hydrocarbon degradation gene clusters, illustrating the diversity of operon organization

Strain	Location	Substrate	Gene	Encoded protein or function	Reference
<i>Pseudomonas putida</i> strains	Plasmid	Naphthalene (upper pathway)	<i>nahAa</i>	Reductase	568
			<i>nahAb</i>	Ferredoxin	679
			<i>nahAc</i>	Iron sulfur protein large subunit	485
			<i>nahAd</i>	Iron sulfur protein small subunit	
			<i>nahB</i>	<i>cis</i> -Naphthalene dihydrodiol dehydrogenase	
			<i>nahF</i>	Salicylaldehyde dehydrogenase	
			<i>nahC</i>	1,2-Dihydroxynaphthalene oxygenase	
			<i>nahE</i>	2-Hydroxybenzalpyruvate aldolase	
			<i>nahD</i>	2-Hydroxychromene-2-carboxylate isomerase	
			<i>nahG</i>	Salicylate hydroxylase	
			<i>nahI</i>	Chloroplast-type ferredoxin	
			<i>nahH</i>	Catechol oxygenase	
			<i>nahI</i>	2-Hydroxymuconic semialdehyde dehydrogenase	
			<i>nahN</i>	2-Hydroxymuconic semialdehyde dehydrogenase	
			<i>nahL</i>	2-Oxo-4-pentenoate hydratase	
			<i>nahO</i>	4-Hydroxy-2-oxovalerate aldolase	
<i>Pseudomonas putida</i> NCIB9816	Plasmid	Naphthalene	<i>nahM</i>	Acetaldehyde dehydrogenase	547
			<i>nahK</i>	4-Oxalocrotonate decarboxylase	
			<i>nahJ</i>	2-Hydroxymuconate tautomerase	
			<i>nahR</i>	Induced by salicylate	
		Regulator for both operons			
<i>Pseudomonas</i> sp. strain C18	Plasmid	Dibenzothiophene Naphthalene phenanthrene	<i>ndoA</i>	Naphthalene-dioxygenase genes (these 3 genes correspond to NahAb,-c, and-d listed above)	355
			<i>ndoB</i>		
			<i>ndoC</i>		
			<i>doxA</i>	Naphthalene dioxygenase	
			<i>doxB</i>	DoxA, -B, -D correspond to NahAb, -c, and-d listed above	
			<i>doxD</i>		
			<i>doxE</i>	<i>cis</i> -Naphthalene dihydrodiol dioxygenase	
			<i>doxF</i>	Salicylaldehyde dehydrogenase	
			<i>doxG</i>	1,2-Dihydroxynaphthalene dioxygenase	
<i>Pseudomonas</i> sp. strain U2	Plasmid	Naphthalene	<i>doxH</i>	Isomerase (interchangeable with <i>doxI</i> ?)	205
			<i>doxI</i>	Hydratase-aldolase	
			<i>doxJ</i>	Isomerase	
<i>Burkholderia</i> sp. strain RP007	Plasmid	Naphthalene phenanthrene	<i>nagAa</i>	Ferredoxin reductase	364
			<i>nagG</i>	Subunit of salicylate 5-hydroxylase with Rieske-type iron-sulfur centre	
			<i>nagH</i>	Subunit of salicylate 5-hydroxylase	
			<i>nagAb</i>	Ferredoxin	
			<i>nagAc</i>	Large dioxygenase subunit	
			<i>nagAd</i>	Small dioxygenase subunit	
			<i>nagB</i>	Naphthalene <i>cis</i> -dihydrodiol dehydrogenase	
			<i>nagF</i>	Salicylaldehyde dehydrogenase	

<i>Pseudomonas putida</i> OUS82	Chromosome	Naphthalene Phenanthrene A variety of homo-hetero-, and monocyclics converted to phenols	<i>phnAc</i> <i>phnAd</i> <i>phnB</i>	Large dioxygenase subunit (Rieske-type [2Fe-2S]) Small dioxygenase subunit Dihydrodiol dehydrogenase	333 601
			<i>pahAa</i> <i>pahAb</i> <i>pahAc</i>	Ferredoxin reductase Ferridoxin Large subunit of iron-sulfur protein	
			<i>pahAd</i> <i>pahB</i> <i>pahC</i> <i>pahD</i> <i>pahE</i> <i>pahF</i>	Small subunit of iron-sulfur protein <i>cis</i> -Dihydrodiol dehydrogenase Dioxygenase Isomerase Hydratase-aldolase Dehydrogenase	
<i>Pseudomonas stutzeri</i> AN10	Chromosome	Naphthalene 2-Methylnaphthalene	<i>nahG</i> <i>nahW</i>	Salicylate 1-hydroxylase Salicylate 1-hydroxylase (outside <i>meta</i> -cleavage transcriptional unit)	63
<i>Nocardia</i> sp. strain KP7	Chromosome	Phenanthrene	<i>phdA</i> <i>phdB</i> <i>phdC</i> <i>phdD</i> <i>phdK</i>	Alpha subunit of dioxygenase Beta subunit of dioxygenase Ferredoxin Ferredoxin reductase 2-Carboxybenzaldehyde dehydrogenase	533
<i>Rhodococcus</i> sp. strain 124	Chromosome	Naphthalene Toluene Indene	<i>nidA</i> <i>nidB</i> <i>nidC</i> <i>nidD</i>	Naphthalene-inducible dioxygenase system Dioxygenase small subunit <i>cis</i> -Dihydrodiol dehydrogenase Putative aldolase	615
<i>Mycobacterium</i> sp. strain PYR-1	Chromosome	Anthracene, Phenanthrene Fluoranthene Pyrene, benzo [a]pyrene, 1-nitropyrene	<i>nidD</i>  <i>nidB</i> <i>nidA</i>	Aldehyde dehydrogenase  Small subunit of dioxygenase Large subunit of dioxygenase	318
<i>Sphingomonas paucimobilis</i> var. EPA505		Phenanthrene Anthracene, benzo[b]fluoranthene Naphthalene Fluoranthene, pyrene Intermediate catabolites	<i>pbbA</i> <i>pbbB</i>  <i>pbbC</i> <i>pbbD</i>	Ring fission dioxygenase Rieske-type ferridoxin subunit of multicomponent dioxygenase  Hydratase-aldolase Pyruvate phosphate dikinase	587

to similar substrate specificities, as Yang et al. (677) found that *P. putida* NCIB 9816 clones were able to produce metabolites from naphthalene, fluorine, and phenanthrene, while only naphthalene metabolites were detected from a homologous NAH7 clone.

In addition, with respect to PAH metabolism, novel gene sequences and gene orders have been observed in a variety of strains, including *Burkholderia* sp. strain RP007, *phnFECDAC AdB* (364); *Pseudomonas* sp. strain U2, *nagAaGHAbAcAdBF* (205); *Rhodococcus* sp. strain I24, *nidABCD* (615); *Mycobacterium* sp. strain PYR1, *nidDBA* (318); and *Nocardioles* sp. strain KP7, *phdABCD* (542). Sequence diversity, and the fact that naphthalene catabolic genes have now been found on the chromosome as well as on plasmids indicate that lateral gene transfer and genetic recombination may have played an important role in the development of these versatile metabolic pathways (63, 64, 205, 364, 542). For example, the *phn* locus has similarities to both *nah* and *bph* genes in *Burkholderia* sp. strain RP007 (364), while the chromosomally encoded *nah* upper and lower pathways in *Pseudomonas stutzeri* AN10 appear to have been recruited from other organisms and recombined. In fact, two entire *nah* upper pathways may exist in this strain (63, 64).

Thus, not only are new gene sequences being found for PAH metabolism, but strains possessing multiple genes for similar enzymes are being detected. Ferrero et al. (189) recently showed, while studying *Pseudomonas* spp. isolated from the western Mediterranean, that single strains can have two distinct *nahAc*-like genes as well as other genes of the upper *nah* pathway. With respect to the lower pathway, Bosch et al. (63) found two distinct genes for salicylate 1-hydroxylase, the flavoprotein monooxygenase that converts salicylate to catechol, on the chromosome of *P. stutzeri* AN10. While the *nahG* gene was found in the *meta*-cleavage pathway transcriptional unit, the novel *nahW* was found close to but outside of this unit. Both are induced upon exposure to salicylate and have broad substrate specificities, but *nahW* is missing the conserved flavin adenine dinucleotide-binding site (GXGXG) normally found in these hydroxylases. This is the first example of two isofunctional salicylate hydroxylases in one strain, and it will be interesting to discover if the combination of genes from various catabolic routes is a widespread phenomenon.

This type of metabolic expansionism is exemplified by *Sphingomonas yanoikuyae* B1, which has recruited, modified, and reorganized genes to obtain catabolic pathways for naphthalene, phenanthrene, anthracene, biphenyl, toluene, and *m*- and *p*-xylene. In this case, *nah*, *bph*, and *xyl* genes are present but are not arranged in three distinct operons (215, 330, 692). Indeed, this gene clustering may be typical of *Sphingomonas* spp. capable of degrading aromatic compounds. Romine et al. (519, 520) sequenced the pNL1 (≈184 kb) plasmid of *Sphingomonas aromaticivorans* F199, which is capable of degrading toluene, xylenes, salicylate, biphenyl, dibenzothiophene, fluorene, and benzoate. In this plasmid, at least 13 gene clusters are predicted to encode all of the necessary enzymes. In addition, seven three-component oxygenases with components spread over six gene clusters have been predicted.

Beyond the genes known to participate directly in PAH metabolism, genes that may provide important support functions are being described. *Sphingomonas paucimobilis* var. EPA500, a strain able to use fluoranthene, naphthalene, and

phenanthrene as sole carbon and energy sources, has *pbhD*, a gene encoding pyruvate phosphate dikinase homologous to *ppdK* that is known to be involved in glucose uptake in prokaryotes and plants. If *pbhD* is disrupted, fluoranthene metabolism is interrupted. While the gene function is not clear, it is possible that it is involved in the uptake of fluoranthene catabolites that leak from the cell (587). Another example is the *katG* gene in *Mycobacterium* sp. strain PYR-1, which encodes an 81-kDa catalase-peroxidase induced upon exposure to pyrene (651). This enzyme may protect the dioxygenase from oxidative inactivation by exogenous oxidation or by removing H<sub>2</sub>O<sub>2</sub> generated endogenously during PAH metabolism (375, 426, 651). Grimm and Harwood (226, 227) recently found *nahY* on the NAH7 catabolic plasmid of *P. putida* G7, which encodes a membrane protein that may be a chemoreceptor for naphthalene or naphthalene metabolites.

In order to move towards a better understanding of the diversity of PAH metabolism in the ecosystem, research should be directed towards genera other than mesophilic pseudomonads. This will allow a variety of research questions to be addressed: what impact different genera have on PAH metabolism in the environment; what and how pathways should be encouraged in active bioremediation systems; and what relationship exists between ecosystem properties and PAH metabolism.

To start, synergistic and antagonistic interactions between PAHs of both high and low molecular weights are being investigated. For example, Molina et al. (433) observed that, for both a mixed culture and *Mycobacterium* sp. strain M1, cross-acclimation occurred between phenanthrene and pyrene metabolism in that pyrene-grown cells did not require new protein synthesis to degrade phenanthrene. On the other hand, neither naphthalene nor anthracene resulted in induction or inhibition of pyrene mineralization. Samanta et al. (537) found that phenanthrene mineralization increased in two strains when fluorine, fluoranthene, and pyrene mixtures were added, while mineralization was not affected in two other strains. In this case, a consortium of the four strains did not enhance phenanthrene mineralization, as has been observed in other studies with defined bacterial and bacterial-fungal consortia (61, 67, 101, 616).

Inhibition may also occur, presumably due to competition for enzymes involved in oxidation or transport, accumulation of by-products resulting in cytotoxicity, and blockage of enzyme induction (66, 295, 590, 564). Determining which mechanism is important in any given situation can be complicated by the presence of metabolites from the different PAHs. The pyrene metabolite *cis*-4,5-dihydro-4,5-dihydroxy pyrene inhibited phenanthrene metabolism in *Pseudomonas saccharophila* strain P15 and *Sphingomonas yanoikuyae* R1 but had little effect on *Pseudomonas stutzeri* P16 and *Bacillus cereus* P21 (313). In addition, the above metabolite and its oxidation product, pyrene-4,5-dione, inhibited benzo[a]pyrene mineralization in the sensitive strains. In a follow-up study, the strains were found to form the dead-end product fluoranthene-2,3-dione as a cometabolic product of fluoranthene when grown on phenanthrene. Phenanthrene removal was inhibited by this metabolite in *Sphingomonas* sp. strain R1 but not in the three other strains studied. Mineralization of benz[a]anthracene, benzo[a]pyrene, and chrysene was also inhibited in R1, while only benzo-

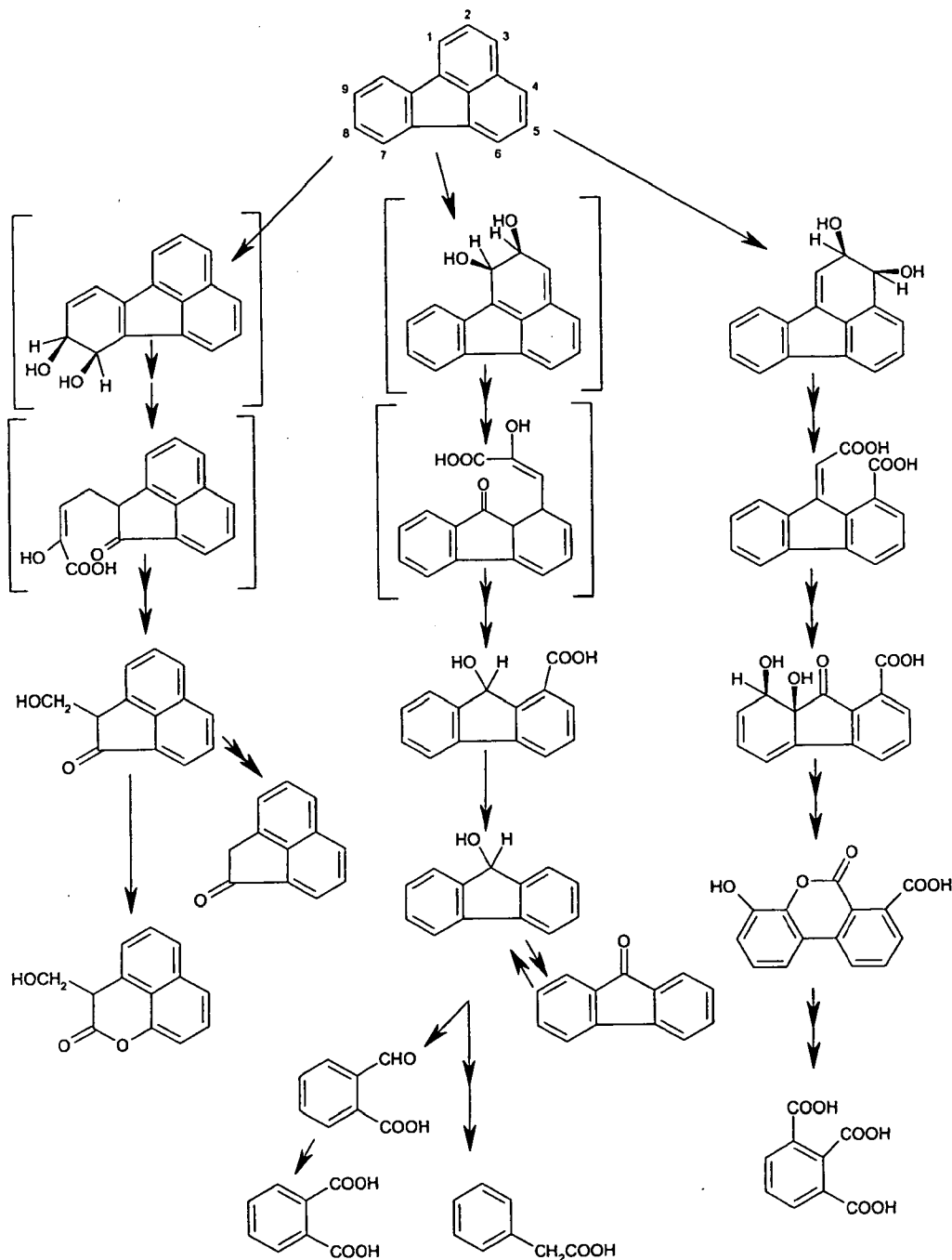


FIG. 3. Bacterial fluoranthene biodegradation pathways, illustrating microbial metabolic diversity with respect to high-molecular-weight PAHs. Intermediates in brackets have not yet been identified.

[a]pyrene metabolism in P15 was affected. Cytotoxicity was partly responsible for the observed inhibition (314). Thus, depending on the strains, transformation products from one PAH may affect the removal of other PAHs (295, 112). Overall, induction effects in complex mixtures may be as important as diauxic effects (49, 304, 305, 418).

Understanding how a metabolite may interact with a specific receptor or enzyme requires knowledge of what metabolites are formed and how persistent they are in the environment.

Indeed, the number of known metabolites from both low- and high-molecular-weight PAHs is increasing as more researchers apply techniques such as high-resolution gas chromatography-mass spectroscopy and nuclear magnetic resonance in their studies. Recent studies with members of the mycobacteria, ubiquitous soil microorganisms with versatile metabolic abilities, illustrate the diversity of PAH metabolic pathways.

For example, Grund et al. (230) noted that *Rhodococcus* sp. strain B4, whose naphthalene metabolic pathway was not in-

duced by salicylate, the normal inducer of the NAH7 pathway, oxidized salicylate to gentisate rather than catechol. More recently, Dean-Ross et al. (144) described a *Rhodococcus* sp. that metabolizes anthracene to 1,2-dihydroxyanthracene and then to either 3-(2-carboxyvinyl)naphthalene-2-carboxylic acid or 6,7-benzocoumarin. The second product is from the *meta*-cleavage pathway found in both gram-positive and gram-negative bacteria, while the first product is from a novel *ortho*-pathway, to date only identified in gram-positives (22, 437, 641). In gram-negatives, novel metabolic pathways for low-molecular-weight PAHs, such as phenanthrene and fluorene, have been recently described as well (100, 537).

The number of strains known to utilize four-ring PAHs as sole carbon and energy sources, even in the absence of cofactors or surfactants, and those known to cometabolize PAHs with more than four rings has increased greatly in the last 10 years. Along with this, a myriad of metabolic pathways have been proposed, as documented by Kanaly and Harayama (301) for a variety of high-molecular-weight PAHs in bacteria, and by Juhasz and Naidu (294), who focused on microbial metabolism of benzo[*a*]pyrene. In the short time since these reviews appeared, more examples of novel metabolic pathways and cooxidation products have been described. For example, Rehmann et al. (507) outlined a new pathway for fluoranthene metabolism in *Mycobacterium* sp. strain KR20, whereby initial dioxygenation commences at the 2,3 position (Fig. 3). Kazunga et al. (314) identified fluoranthene-2,3-dione and fluoroanthene-1,5-dione as dead-end metabolites from fluoranthene during growth on phenanthrene in *Pseudomonas saccharophila* strain P15, *Sphingomonas yanoikuyae* strain R1, *Pseudomonas stutzeri* P16, and *Bacillus cereus* strain P2. These metabolites are not likely to be intermediates of fluoranthene metabolism, but instead are probably autooxidation products of the corresponding *o*-dihydroxy metabolites.

It is becoming evident that many strains employ monooxygenases or both monooxygenases and dioxygenases for the metabolism of single-ring PAHs (20, 437, 614, 641). In addition, classic dioxygenase enzymes such as the multicomponent naphthalene dioxygenase can catalyze monohydroxylation, dihydroxylation, desaturation, O- and N-dealkylation, and sulfoxidation reactions against a wide variety of monocyclic and heterocyclic compounds (217, 369, 509, 553). Site-directed mutagenesis of naphthalene dioxygenase indicates that slight changes in amino acid sequence can have profound effects on reaction regio- and stereospecificity (681). Questions related to enzyme functionality and the evolution of similar naphthalene dioxygenases in different genera (e.g., *Pseudomonas* and *Rhodococcus*) will be answered as more enzymes are purified and characterized (93, 237, 310, 368, 414, 361, 472, 591, 592).

Overall, the broad PAH-degrading capabilities in many strains may be attributed to relaxed initial enzyme specificity for PAHs (low and high molecular weight and methyl substituted), the presence of multiple oxygenases, and the presence of multiple metabolic pathways or multiple genes for isofunctional pathways (83, 112, 160, 249, 220, 330, 396, 399, 418, 437, 519, 520, 532, 641, 677). Finally, the presence of both alkane and aromatic compound-degrading genes within single strains appears to be common (120, 301, 576, 578, 641, 662).

How these various metabolic routes are controlled at the genetic level and how they compete for a substrate is still a

major question. This is especially evident when novel dead-end metabolites, such as the methoxylated 1-methoxy-2-hydroxyanthracene from anthracene metabolism (641) and the dicarboxylic acid 6,6'-dihydroxy-2,2'-biphenyl dicarboxylic acid from pyrene metabolism (437), are detected with strains simultaneously employing multiple degradative routes for a single substrate. This is also the case in strains that have degradative pathways for multiple aromatic substrates (588, 519). For example, in *Sphingomonas aromaticivorans* strain F199, induction studies have indicated that naphthalene and toluene mineralization may be higher in the presence of both substrates, as greater gene expression can be achieved (519).

### Anaerobic Hydrocarbon Metabolism

Anaerobic metabolism is a vital process with respect to petroleum hydrocarbon biodegradation and bioremediation and, given the unique biochemistry now being uncovered, is also vital with respect to biomimetic catalyst development. Currently, we are in a period of rapid expansion with quality, convention-shattering work being released at an exciting pace. This is evidenced by the number of reviews being published in the area after 10 years of accelerated discovery (203, 243, 250, 271, 390, 482, 581, 664). Given the scope of the current reviews, a brief overview of anaerobic hydrocarbon metabolism will be given, with mention of some new advances since Widdel and Rabus published their review in 2001 (664).

Work with microbial consortia in the field, in enrichment cultures, and in microcosms has illustrated that hydrocarbons such as toluene (171, 358), alkylbenzenes including *m*-, *o*-, and *p*-xylene and trimethylbenzenes (39, 111, 235, 481), benzene (90, 312, 521), naphthalene and phenanthrene (50, 124, 421, 686), methylnaphthalene and tetralin (20, 23), >C<sub>6</sub> *n*-alkanes (18, 96, 168, 575), branched alkanes (72, 73), and hydrocarbon mixtures (228) can be metabolized under anaerobic conditions. These reactions may take place under Fe(III)-reducing, denitrifying, and sulfate-reducing conditions, by anoxygenic photosynthetic bacteria, or in syntrophic consortia of proton-reducing and methanogenic bacteria. Other terminal electron acceptors shown to be used during anaerobic hydrocarbon metabolism include manganese oxides (357, 358), soil humic acids and the humic acid model compound anthraquinone-2,6-disulfonate (105), and fumarate in a fermentative oxidation process (420). Mixed-culture work continues as enhanced bioremediation strategies are tested (17, 530) and new metabolites are described (23, 172, 421, 687).

More recently, the number of pure cultures shown to metabolize various hydrocarbons with different electron acceptors has increased (Table 2). This diverse set of bacteria (no fungi have been studied to date), including members of the  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -subclasses of the proteobacteria, form an excellent framework from which to elucidate the underlying biochemical and molecular mechanisms driving anaerobic hydrocarbon metabolism.

Toluene has been the most studied hydrocarbon with respect to enzymatic and genetic characterizations in the denitrifying bacteria *Azoarcus* sp. strain T, *Thauera aromatica* strain K172, and *Thauera* sp. strain T1 (2, 52, 53, 54, 58, 135, 136, 250, 255, 349, 378, 379). In the proposed pathway, fumarate addition to toluene is mediated by benzylsuccinate synthase to form ben-

TABLE 2. Petroleum hydrocarbon-degrading anaerobic bacteria

Organism	Hydrocarbon(s) used	Reference
Anoxygenic photoheterotrophic bacterium <i>Blastochloris sulfoviridis</i> ToP1	Toluene	685
Denitrifying bacteria		
<i>Azoarcus</i> sp. strain EB1	Ethylbenzene	38
<i>Azoarcus</i> sp. strain T	Toluene, <i>m</i> -xylene	152
<i>Azoarcus toluityticus</i> Td15	Toluene, <i>m</i> -xylene	204
<i>Azoarcus toluityticus</i> To14	Toluene	690
<i>Dechloromonas</i> sp. strain J1	Benzene, toluene	125
<i>Dechloromonas</i> sp. strain RCB	Benzene, toluene	125
<i>Pseudomonas</i> sp. strain NAP-3	Naphthalene	517
Strain EbN1	Ethylbenzene, toluene	495
Strain HdN1	C <sub>14</sub> -C <sub>20</sub> alkanes	168
Strain HxN1	C <sub>6</sub> -C <sub>8</sub> alkanes	168
Strain M3	Toluene, <i>m</i> -xylene	256
Strain mXyN1	Toluene, <i>m</i> -xylene	495
Strain OcN1	C <sub>8</sub> -C <sub>12</sub> alkanes	168
Strain PbN1	Ethylbenzene, propylbenzene	495
Strain pCyN1	<i>p</i> -Cymene, toluene, <i>p</i> -ethyltoluene	238
Strain pCyN2	<i>p</i> -Cymene	239
Strain T3	Toluene	256
Strain ToN1	Toluene	495
<i>Thauera aromatica</i> K172	Toluene	16
<i>Thauera aromatica</i> T1	Toluene	181
<i>Vibrio</i> sp. strain NAP-4	Naphthalene	517
Fe(III)-reducing bacteria		
<i>Geobacter grbiciae</i> TACP-2 <sup>T</sup>	Toluene	123
<i>Geobacter grbiciae</i> TACP-5	Toluene	123
<i>Geobacter metallireducens</i> GS15	Toluene	391
Sulfate-reducing bacteria		
<i>Desulfobacula toluolica</i> To12	Toluene	497
<i>Desulfobacterium cetonicum</i>	Toluene	238
Strain AK-01	C <sub>13</sub> -C <sub>18</sub> alkanes	574
Strain Hxd3	C <sub>12</sub> -C <sub>20</sub> alkanes, 1-hexadecene	4
Strain mXyS1	Toluene, <i>m</i> -xylene, <i>m</i> -ethyltoluene, <i>m</i> -cymene	238
Strain NaphS2	Naphthalene	208
Strain oXyS1	Toluene <i>o</i> -xylene, <i>o</i> -ethyltoluene	238
Strain Pnd3	C <sub>14</sub> -C <sub>17</sub> alkanes, 1-hexadecene	4
Strain PRTOL1	Toluene	54
Strain TD3	C <sub>6</sub> -C <sub>16</sub> alkanes	531

zylsuccinate. Following this unusual addition reaction, a series of modified  $\beta$ -oxidation reactions are thought to convert benzylsuccinate to benzyl-CoA (52, 53, 58), which is a central intermediate in the anaerobic degradation of aromatic compounds (243).

Benzylsuccinate synthase has been purified from *Azoarcus* sp. strain T and *T. aromatica* strain K172 and is characterized as a  $\alpha_2\beta_2\gamma_2$  heterohexamer with a flavin cofactor but no iron-sulfur clusters (54, 378) and represents a new class of glycol radical-containing enzymes (350). Succinyl-CoA:(*R*)-benzylsuccinate CoA-transferase, which activates (*R*)-benzylsuccinate to 2-(*R*)-benzylsuccinyl-CoA, has also been purified from strain *Thauera aromatica* K172 (380).

The genes encoding benzylsuccinate synthase have been cloned and sequenced in *Azoarcus* sp. strain T (2), *T. aromatica* strain K172 (378), and *T. aromatica* strain T1 (135, 136, 137, 378). In strain *T. aromatica* K172, the *bbs* (beta-oxidation of benzylsuccinate) operon contains *bbsDCABE*, with *bbsCAB* encoding the  $\gamma$ ,  $\alpha$ , and  $\beta$  subunits of benzylsuccinate synthase, a region with significant homology to the *tutFDG* genes in

strain T1 (136, 255, 378). The genes encoding the putative activating enzyme (*bssD* and *tutE*) are found upstream and also show homology in the two strains. *BssE* in K172 may be an ATP-dependent chaperone for assembly or deactivation of benzylsuccinate synthase (255). In contrast to K172 and T1, strain T mineralizes both toluene and *m*-xylene. In this case, expression of the *bssDCABE* operon is required for growth on both substrates (2).

Similar operons may be present in other strains, as the novel benzylsuccinate synthase reaction, catalyzing the addition of fumarate to toluene (110, 181), may also be involved in the metabolism of xylenes (349, 444, 445), alkyl-naphthalenes (20, 23), *n*-hexadecane (497), and *n*-dodecane (351). For example, dodecylsuccinic acids were detected from a sulfate-reducing enrichment culture growing on *n*-dodecane (351), and an *n*-hexane-utilizing denitrifying bacterium with a protein similar to *BssC* has been isolated from the toluene-degrading denitrifying bacteria (664). In addition, the metabolites (1-methylpentyl)succinate and (1-ethylbenzyl)succinate from the anaerobic metabolism of *n*-hexane by a denitrifying strain indicate a C-2

and a C-3 addition of fumarate, analogous to the toluene activation reaction (497). The (1-methylpentyl)succinate is then converted to a CoA thioester prior to rearrangement to (2-methylhexyl)malonyl-CoA and degradation by conventional  $\beta$ -oxidation (666). Thus, it appears that the fate of the alkylsuccinates produced is probably fatty acid metabolism (5, 574, 666).

For ethylbenzene, oxidation under denitrifying conditions appears to commence with a dehydrogenation by ethylbenzene dehydrogenase to produce 1-phenylethanol followed by oxidation to acetophenone (39, 108, 291, 495, 496). Ethylbenzene dehydrogenase has been isolated from both *Azoarcus* sp. strains EB1 (292) and EbN1 (335). In both cases, the enzyme is an  $\alpha\beta\gamma$ -Mo-Fe-S heterotrimer. Johnson et al. (292) sequenced *ebdA*, encoding the  $\alpha$ -subunit containing a molybdopterin-binding domain; *ebdB*, encoding the  $\beta$ -subunit containing several 4Fe-4S binding domains; and *ebdC*, encoding the  $\gamma$ -subunit, a potential membrane anchor subunit. Knienmeyer and Heider (334) isolated the  $\text{NAD}^+$ -dependent secondary alcohol dehydrogenase (*S*)-1-phenylethanol dehydrogenase, which catalyzes acetophenone formation in *Azoarcus* sp. strain EbN1. Analogous reactions are believed to occur for *n*-propylbenzene (495), while for sulfate-reducing bacteria the metabolic pathway may be similar to that of toluene metabolism, as (1-phenylethyl)succinate has been detected in enrichment cultures (172). It is of interest that *Azoarcus* sp. strain EbN1 also degrades toluene, but via benzylsuccinate (496).

Two- and three-ring PAHs may also be metabolized under anaerobic conditions. For naphthalene, activation proceeds via carboxylation to form 2-naphthoate in sulfate-reducing (208, 438) and denitrifying (517) bacteria. Carboxylation has also been observed for phenanthrene added to a sulfidogenic culture (686). Alkyl naphthalenes appear to be activated by a mechanism similar to that of toluene, as naphthyl-2-methylsuccinate has been detected in sulfate-reducing enrichment cultures exposed to 2-methylnaphthalene (20).

Recently, Annweiler et al. (23) proposed that, with a sulfate-reducing enrichment culture, naphthalene, 2-methylnaphthalene, and tetralin (1,2,3,4-tetrahydronaphthalene) are all degraded, with 2-naphthoic acid being the central intermediate in a pathway analogous to the benzyl-CoA pathway for monoaromatic compounds. Further degradation occurs through saturated compounds with cyclohexane ring structures (also see 687). They have also found that a sulfate-reducing enrichment culture cometabolized benzothiophene when grown with naphthalene. While activity was not very high, perhaps because of inhibition, toxicity of benzothiophene or metabolites, or benzothiophene being a poor substrate, the products formed (2- and 5-carboxybenzothiophene) indicated that the initial enzyme could nonspecifically attack either the benzene or thiophene ring. As for naphthalene, the  $\text{C}_1$  unit was derived from bicarbonate, as revealed in  $^{13}\text{C}$  radiolabeling experiments (22). In similar experiments with  $^{13}\text{C}$  bicarbonate and 2- $^{14}\text{C}$  methylnaphthalene, the formation of 2-naphthoic acid via methyl group oxidation was observed in a sulfate-reducing consortium. Also, the presence of 2-methylnaphthalenes suggests an alternative metabolic pathway (594).

To date, the mechanism of benzene activation leading to its anaerobic degradation has not been elucidated because no pure cultures have yet been isolated for study. Recently, two

*Dechloromonas* strains (RCB and JJ) of the  $\beta$ -proteobacteria that mineralize benzene with nitrate as the electron acceptor have been isolated (123), and elucidating the genetics and biochemistry of this metabolism is an area that deserves attention.

The diversity and unique properties of the anaerobic hydrocarbon-utilizing bacteria are areas that are in need of more work. While difficult, greater focus on isolating and characterizing the enzymes involved in anaerobic hydrocarbon metabolism is required. Furthermore, uptake, efflux, and chemotaxis, areas only recently explored for aerobes, are topics so far untouched in the anaerobic realm. A balanced shift from molecular biology back to enzymology and protein biochemistry is a move that would benefit the understanding of hydrocarbon metabolism in all areas.

### BEHAVIORAL AND PHYSIOLOGICAL RESPONSES TO HYDROCARBONS

The molecular and biochemical basis of microbial behavior and physiological responses to hydrocarbons and the impact of these responses on bioremediation have been neglected until very recently. Relatively speaking, the metabolic pathways driving the activation of hydrocarbons into central metabolic pathways are well understood, while behaviors and responses are not appreciated beyond a general observational level. However, these phenomena are essential for allowing hydrocarbon-metabolizing organisms to avoid toxic effects, to access poorly soluble substrates, and, in some cases, to bring very large substrates into the cell. This section will examine some of the recent research into the biochemical mechanisms that control responses to hydrocarbons in an effort to suggest that responses such as changes in membrane architecture, active uptake and efflux, and chemotaxis are all of paramount importance and, in some cases, may be coordinately controlled in order to allow metabolism to take place.

#### Membrane Alterations, Uptake, and Efflux

Given the hydrophobic nature of the area between the monolayers of the cytoplasmic membrane and, in gram-negative bacteria, of the outer membrane, it is not surprising that lipophilic molecules such as hydrocarbons partition there. In 1995, Sikkema et al. (566) published an extensive review on the mechanisms of membrane toxicity of hydrocarbons for a variety of organisms. They outlined the toxicity of lipophilic compounds, including hydrocarbons (alkanes, cyclic hydrocarbons), alcohols, phenols, and other antimicrobials. Briefly, hydrocarbons tend to reside in the hydrophobic area between membrane monolayers in the acyl chains of phospholipids, with partitioning being related to the octanol-water partition coefficient of the lipophilic compound. Hydrocarbon insertion alters membrane structure by changing fluidity and protein conformations and results in disruption of the barrier and energy transduction functions while affecting membrane-bound and embedded enzyme activity (143, 251, 566).

In terms of general stress responses, bacteria may form biofilms, alter their cell surface hydrophobicity to regulate their partitioning with respect to hydrocarbon-water interfaces or, in gram-negative bacteria, gain protection from hydrophilic lipo-

polysaccharide components that offer high transfer resistance to lipophilic compounds. In addition, energy-dependent repair mechanisms may be used to compensate for losses in membrane integrity resulting from the partitioning of lipophilic compounds. For example, membrane fluidity can be decreased through increased membrane ordering by affecting *cis/trans* phospholipid isomerizations, by decreasing unsaturated fatty acid content, and by altering phospholipid head groups (297, 501, 566, 617, 659). These changes may be associated with an overall increase in phospholipid content and increased phospholipid biosynthesis in solvent-stressed cells (484).

These alterations serve to produce a physical barrier to the intercalation of hydrocarbons in membranes, thus offsetting the passive influx of hydrocarbons into the cell. It is generally believed that hydrocarbons interact with microorganisms nonspecifically and move passively into the cells (45). Of course, hydrocarbon-degrading microorganisms must necessarily come in contact with their substrates before any transport, either active or passive, may take place. Traditionally, three modes of hydrocarbon uptake are cited to describe how hydrocarbon-metabolizing organisms come in contact with their substrates. However, since uptake implies an active movement of substrate across the cell membrane, a more accurate nomenclature for the initial stages of cell-substrate interaction may be hydrocarbon access (631). While microorganisms may contact water-solubilized hydrocarbons, decreasing solubility with increasing molecular weight is restrictive (91). Two additional, perhaps more widespread modes of hydrocarbon accession are direct adherence to large oil droplets and interaction with pseudosolubilized oil (67). For example, Van Hamme and Ward (631) described a *Rhodococcus* strain that grew directly on crude oil droplets and could be removed with the addition of exogenous chemical surfactant, while a *Pseudomonas* strain required surfactant-solubilized oil to efficiently access hydrocarbons. In *P. aeruginosa*, hydrocarbon solubilization and micellar transport control hexadecane biodegradation during biosurfactant-enhanced growth (552). Similarly, encapsulating solid *n*-C18 and *n*-C36 in liposomes increased growth and biodegradation by a *Pseudomonas* sp., indicating that cell-liposome fusion may deliver encapsulated hydrocarbons to membrane-bound enzymes (427).

Only a limited number of studies conclusively indicate that active hydrocarbon uptake into bacterial cells occurs. Naphthalene uptake by *P. putida* PpG1 appears to be nonspecific, as there is no inhibition by protein inhibitors or iodacetamine and no requirement for specific naphthalene degradation gene expression (45). Similarly, phenanthrene uptake by *Pseudomonas fluorescens* LP6a appears to be passive, in contrast to the observed energy-dependent phenanthrene efflux (84). With respect to active transport, proton motive force uncouplers have been shown to apparently decrease both *n*-hexadecane (46) and naphthalene (660) uptake, which could indicate that energy-dependent uptake is important in some strains. In these two studies, the fact that the strains being studied could metabolize the substrates over the long incubation times complicates the separation of phenomena related to transport, metabolism, and growth. Probably the best observational evidence for energy-dependent alkane uptake is the case of *Rhodococcus erythropolis* S+14He, which preferentially accumulates *n*-hexadecane from hydrocarbon mixtures (327).

Recently, Story et al. (587) identified a gene (*pbhD*) in *Sphingomonas paucimobilis* var. EPA505 that is necessary for fluoranthene metabolism and has homology to the gene pyruvate phosphate dikinase (*ppdK*), a gene involved in glucose uptake in prokaryotes and plants. The authors postulated that *pbhD* may be involved in the uptake of fluoranthene catabolites that leak from the cell, although no experiments were performed to verify this. Even though direct molecular evidence for active uptake has not been presented, it would not be surprising to find energy-dependent pumps that transport hydrocarbons into the cell. The presence of hydrocarbon inclusions, of both pure and partially oxidized alkanes, for example (46, 274), indicates that these substrates can be accumulated against a concentration gradient, presumably an energy-dependent process. In addition, as has been observed for 2,4-dichlorophenoxyacetate (244) and 4-hydroxybenzoate (245) metabolism, uptake and chemotaxis may be coordinately controlled at the molecular level.

**Mechanisms of tolerance.** While an undisputed molecular mechanism for active hydrocarbon uptake is not yet available, excellent descriptions of active hydrocarbon efflux from bacterial cells have been presented in the last 7 years. In their review, Sikkema et al. (566) stated that "there is no precedent why active excretion systems should not play a role in lowering the concentrations in the cytoplasmic membrane (and cytoplasm) of toxic lipophilic molecules." Since that time, two *Pseudomonas putida* strains (DOT-T1E and S12) have been characterized in great detail, both physiologically and genetically, with respect to their ability to thrive in the presence of hydrocarbons. The most notable advance in this area has been the molecular characterization of active solvent efflux pumps for aromatic hydrocarbons (322, 332, 382, 441, 518).

Ramos et al. (501) isolated *P. putida* DOT-T1E, which metabolizes toluene and is capable of growing in the presence of 90% (vol/vol) toluene. In early studies, DOT-T1E was found to increase membrane rigidity by converting *cis*-9,10-methylene hexadecanoic acid to 9-*cis*-hexadecanoic acid and subsequently to the corresponding *trans* isomer. This short-term response typically occurs in less than 1 min upon exposure to toluene. *P. putida* S12, which does not grow on toluene but can tolerate high levels of organic solvents such as styrene (658) and toluene (659), also exhibits *cis/trans* isomerizations (659). In the long-term (15 to 20 min) exposure, DOT-T1E decreased the amount of phosphatidylethanolamine in the phospholipid polar head groups and increased cardiolipid levels, again increasing membrane rigidity (501). These changes increase lipid ordering to restore membrane integrity and reduce organic solvent partitioning in the membrane. A gene encoding a *cis/trans* isomerase, *cti*, which catalyzes the isomerization of esterified fatty acids in phospholipids (mainly *cis*-oleic acid [C<sub>16:1,9</sub>] and *cis*-vaccenic acid [C<sub>18:1,11</sub>]) has been cloned and sequenced in DOT-T1E.

Null mutants exhibited lower survival rates upon toluene shock. In addition, while a longer lag time was observed when mutants were exposed to toluene in the vapor phase, the growth rates for the mutant and the wild-type strain were similar. Thus, the *cis/trans* isomerization helped prevent cell damage but was apparently not the most important element in solvent resistance. *Cti* is constitutively expressed in DOT-T1E and, as expected, is located in the membrane. The *cti* gene is



also found in nonresistant *P. putida* strains and other *Pseudomonas* species (297).

Toluene tolerance in DOT-T1E was found to be inducible by exposure to toluene in the vapor phase, which led the group to postulate that an active solvent exclusion system and metabolic toluene removal afforded some protection (501). Similarly, resistance to antibiotics and solvents such as ethanol was found to increase in S12 with exposure to toluene but not antibiotics (279). In [<sup>14</sup>C]toluene influx studies, an energy-dependent efflux system was proposed, as less influx was observed in adapted cells, while greater influx was observed in the presence of potassium cyanide, a respiratory chain inhibitor, and *m*-chlorophenylhydrazine, a proton conductor (276). The interruption of toluene metabolism through mutation of the *tod* genes did not affect toluene tolerance in DOT-T1E, suggesting that some other mechanism of tolerance was involved (440). Indeed, active solvent exclusion systems, have been characterized in these two strains.

The *srpABC* (solvent resistance pump) genes of *P. putida* S12 were the first to be cloned and unambiguously shown to be responsible for toluene efflux (322). The pump consists of SrpB (inner membrane transporter), SrpC (outer membrane channel), and SrpA (periplasmic linker protein) and is homologous to the proton-dependent multidrug efflux systems of the resistance/modulation/cell division (RND) family of pumps, which export antibiotics, metals, and oligosaccharides. These pumps have been well reviewed by Paulsen et al. (477).

Induced by aromatic and aliphatic solvents and alcohols, the efflux system encoded by *srpABC* is proton dependent and does not pump antibiotics or other substrates of multidrug resistance pumps (277). Unlike *cis/trans* isomerisations, which can be a general stress response (251), the *srpABC* genes are not induced by extremes of pH, temperature, salt, organic acids, or heavy metals (323). These adaptation mechanisms are energy consuming and have been shown to decrease growth rates and yields while increasing maintenance energy and lag times (278). Presumably, the increased energy consumption may also result from solvent-mediated membrane uncoupling and disruption of energy-transducing proteins.

The first efflux pump in DOT-T1E was found by producing a toluene-sensitive, octanol-tolerant mutant (DOT-T1E-18) by Tn5-*phoA* mutagenesis with a gene knockout homologous to the drug exclusion gene *mexB*, which is a member of the efflux pump family of the resistant modulator type (502). The gene was named *ugB* for toluene tolerance gene. Solvent exclusion testing with 1,2,4-[<sup>14</sup>C]trichlorobenzene showed that increasing toluene concentrations increased the amount of radiolabel in the membranes. In addition, the pump was shown to be specific, as DOT-T1E is sensitive to benzene but not *m*-xylene. Given the fact that the mutant exhibited low levels of survival when toluene was delivered in the vapor phase, it was postulated that at least two efflux pumps were present, one constitutive and one inducible.

Indeed, three toluene efflux pumps have ultimately been found in DOT-T1E (441, 518). This is not without precedent, as *P. aeruginosa* has at least three RND antibiotic efflux pumps, which also accommodate organic solvents: MexAB-OprM, MexCD-OprJ, and MexEF-OprN (381, 382). The first pump in DOT-T1E, *ugABC*, is a constitutive efflux pump controlled by

*ugR*, which produces a transcriptional repressor for the *ugABC* operon, which in turn is controlled by another repressor belonging to the Lrp family of global regulators. In this case, TtgR is expressed at high levels in the presence of toluene, which in turn reduces TtgABC expression (158). The second pump, *ugDEF*, is found adjacent to the *tod* genes and is expressed in response to toluene and styrene. Unlike *ugABC*, *ugDEF* does not appear to efflux antibiotics and is closely related but not identical to the toluene efflux pump *srpABC* of *P. putida* S12.

The third pump, *ugGHI*, is expressed constitutively at high levels from a single promoter and, if grown with toluene, is expressed at higher levels from two promoters: one a constitutive promoter and a second, overlapping, inducible promoter (518). *ugG* encodes the periplasmic lipoprotein that is anchored to the inner membrane and, along with the inner membrane pump encoded by *ugG*, forms the putative translocase. *ugI* encodes the outer membrane protein that may form a channel into the periplasmic space (518). In order to make DOT-T1E sensitive to toluene shock and to eliminate its ability to grow with toluene in the gas phase, mutations had to be introduced in all three pumps. Mutation studies showed that TtgABC and TtgGHI pump toluene, styrene, *m*-xylene, ethylbenzene, and propylbenzene. TtgDEF only removes toluene and styrene.

Overall, it appears that efflux pumps in *Pseudomonas* spp. can be divided into three general groups: those that pump organic solvents, those that pump antibiotics, and those that pump both. Kieboom et al. (321) recently described an active antibiotic efflux pump in S12 (ArpABC) which does not pump solvents. This is in contrast to the MepABC pump in *P. putida* KT2442 (206) and the Mex pumps in *P. aeruginosa* (382), which pump both solvents and antibiotics. Furthermore, much will be gained if efflux pumps for other hydrocarbons and for other microorganisms are studied in detail and compared to known systems. Further research at the protein level will be required for many systems, as comparative studies will help to unravel the factors affecting pump specificity, to understand what forces govern substrate recognition, and to see if and how pump receptors are able to regulate other behaviors such as taxis, the final behavior to be discussed here.

### Taxis

Motile bacteria are able to control their spatial position with respect to various stimuli such as chemicals, light, and redox potential by a variety of mechanisms. Chemotaxis is the response to a stimulus independent of cellular metabolism through chemoreceptors. On the other end of the spectrum, a microorganism may exhibit metabolism-dependent energy taxis, where behavioral responses are to changes in energy levels in the cell and not the stimulus itself. Finally, there are cases when the chemotactic behavior is in response to substrate transport across the membrane (604). The molecular and biochemical nature of these behaviors has been relatively well studied for water-soluble substrates and has only recently been explored in hydrocarbon-degrading bacteria.

One can imagine that movement away from a hydrocarbon plume could reduce toxic effects or that movement towards a

water-insoluble substrate such as naphthalene could be advantageous in poorly mixed field situations. Indeed, Marx and Aitken (410) used a capillary assay (409) to show that *Pseudomonas putida* G7 catalyzed naphthalene degradation at faster rates in unmixed, heterogeneous systems than did mutants deficient in either motility or naphthalene chemotaxis. In mixed systems, the naphthalene degradation rate was identical for the wild-type and mutant strains.

*P. putida* G7 possesses the NAH7 catabolic plasmid for the meta-cleavage of aromatic hydrocarbons (226, 227). The plasmid includes the *nahY* gene, encoding a 538-amino-acid membrane protein whose C terminus resembles that of chemotaxis transducer proteins (i.e., methyl-accepting chemotaxis proteins). This indicates that NahY may be a chemoreceptor for naphthalene or naphthalene metabolites (227), but neither the molecular nature of binding nor the cascade of responses that occur following binding has been studied.

*Pseudomonas putida* RKJ1 possesses an 83-kb plasmid for naphthalene metabolism through salicylate (538). A Nap<sup>-</sup>Sal<sup>+</sup> mutant was chemotactic towards only salicylate, while a Nap<sup>-</sup>Sal<sup>-</sup> mutant exhibited no chemotaxis. This suggests the presence of a metabolism-dependent energy taxis in this strain. Thus, a change in the redox potential or cellular energy level in the cell probably provides the signal for chemotaxis. Alternatively, a membrane-bound or intracellular chemoreceptor may recognize naphthalene or salicylate degradation products.

To date, no reports describing the molecular basis for alkane chemotaxis have appeared. However, van Beilen et al. (627) detected *alkN* in the 9.7-kb region between *alkBFGHJKL* and *alkST* in *P. putida* GPo1, which encodes a protein with 30% sequence similarity to methyl-accepting transducers such as the one found in strain G7 (227). As GPo1 is not very motile, the functionality of the gene is difficult to study.

Overall, taxis in relation to petroleum hydrocarbons has been neglected, and the area is ripe for study. First of all, more examples of tactic behavior to hydrocarbons are required in other genera and with different hydrocarbons in order to appreciate the diversity of responses. Second, when putative chemoreceptors are detected by gene sequencing, systematic studies of purified proteins are required in order to understand the key molecular interactions that take place to allow a cell to detect a particular chemical. Third, the mechanisms by which chemoreceptors translate signals induced by hydrocarbons into cellular responses and their impact on overall cellular biochemistry would allow the integration of this behavior, and all of the behaviors discussed here, into a larger picture of hydrocarbon-metabolizing organisms. Recent developments for the large-scale and nearly real-time monitoring of gene expression in live cells with green fluorescent protein promoter fusions (300, 579) will allow this type of integrating study. Finally, understanding the true role of chemotaxis during remediation needs more attention if we are going to understand the impact of taxis on biofilm formation, substrate access, and avoidance of toxic substances. Recent developments in tracking live bacterial cells with advanced imaging technologies (559) could be combined with gene expression technologies and traditional measurements of hydrocarbon degradation (258) to study these questions.

## MICROBIAL COMMUNITY DYNAMICS

Ecologically, hydrocarbon-metabolizing microorganisms are widely distributed. Difficulties arising during attempts to characterize natural microbial communities impacted by petroleum hydrocarbons are exacerbated by the myriad of individual substrate and metabolite interactions possible. Despite the intricacies, tools are being developed in an attempt to better appreciate microbial abundance and distribution in natural environments in the hopes of associating community structures with ecosystem functions. The rationale for undertaking such analyses includes describing the role of microorganisms in the genesis of petroleum over geological time (398, 465), evaluating the long-term effects of petroleum pollution (386), developing and evaluating waste remediation approaches (298, 565), tracking the enrichment of pathogenic microorganisms during remediation (56, 197), and controlling deleterious microbial activities during petroleum production (165, 166).

Approaches to cataloguing microbial diversity and community function can be broadly divided into culture-dependent and culture-independent methods, both of which may include genetic characterization techniques. Traditional culture-dependent methods are the most familiar and are based on differential morphological, metabolic, and physiologic traits. These include isolation and cultivation on solid media, most-probable-number (MPN)-style liquid assays, and more recently, Biolog substrate utilization plates. Culture-independent methods for community analysis began with direct examination of metabolically active microorganisms with differential stains such as 4',6'-diamidino-2-phenylindole, (INT)-formazan and CTC, fluorescence in situ hybridization, and bulk analysis of total protein banding and phospholipid fatty acid analysis.

With rapid expansions in the field of molecular genetics, a host of PCR-based approaches have emerged to study specific microorganisms or groups of microorganisms and specific genes and to evaluate overall community profiles. Methods to evaluate community profiles include denaturing and temperature gradient gel electrophoresis, ribosomal intergenic spacer analysis, single-strand conformation polymorphism, internal transcribed spacer-restriction fragment length polymorphism, random amplified polymorphic DNA, and amplified ribosomal DNA restriction analysis (317). Recently, developments in the use of DNA microarrays have attracted the attention of environmental microbiologists for more rapid throughput to allow the tracking of thousands of genes at one time (146).

A few examples of community studies involving petroleum applications are discussed here in order to highlight the utilities and limitations of the various methods (Table 3).

## Culture-Based Methods

Traditional culture techniques have yielded valuable information about microbial interactions with hydrocarbons in the environment. However, one must keep in mind that only a small fraction of microorganisms can currently be cultured from environmental samples, and even if a microorganism is cultured, its role in a community and contribution to ecosystem function are not necessarily revealed. This was especially evident in early studies, where catalogues of microorganisms were compiled based on conventional isolation and plating tech-

TABLE 3. Utility and limitations of some community analysis methods

Type	Example	Utility	Limitations
Culture dependent	Plating	Isolates obtained for further study	Only a small proportion of community detected, isolates not necessarily reflective of a specific metabolic function
	MPN	Metabolic function of interest detected	No isolates obtained for further study, selective media may limit proportion of community detected
	Biolog	Overall metabolic activity detected, rapid and easy to use	No isolates obtained for further study, selective media may limit proportion of community detected, may not include substrates of interest, sensitive to inoculum size and incubation effects
Culture independent	Phospholipid fatty acid analysis	Changes in fingerprint can indicate change in community structure	No isolates obtained for further study
	Protein banding	No selection pressure if extracted directly	No measurement of community function, difficult to link fingerprints to specific microbial groups
	Fluorescence in situ hybridization	Spatially visualize specific microorganisms in an environment, no bias from culture media	Not necessarily detecting active microorganisms, laborious technique
	Staining for active microbes	Enumerate live microorganisms, no bias from culture media	Does not differentiate microorganisms with catabolic activity of interest
	RSGP <sup>a</sup>	Quantitative analysis of specific microorganisms in environmental samples, no bias from culture media	Limited to those microorganisms included in the screen
	PCR followed by gel electrophoresis	No bias from culture media, can identify microorganisms by sequencing resolved bands, bulk changes in community structure detected	Differential DNA or RNA extraction from different cells, differential amplification during PCR, no information on activity; no isolates for study
	Probes for specific metabolic genes	Detect genes with function of interest, mRNA detection can reveal information about expression	Limited to known genes, activity cannot be inferred from presence of genes alone
	Promoter-reporter systems	Gene expression detected, treatment effects on total cell function can be monitored	Nature of promoter must be known, easier to apply when whole genome sequences are available, monitors only those strains with reporter genes inserted

<sup>a</sup> RSGP, reverse sample genome probing.

niques. These studies documented a broadly distributed and diverse collection of bacteria, yeasts, and fungi capable of hydrocarbon utilization (29), and similar contemporary investigations continue to catalogue microbial communities from hydrocarbon-impacted environments around the world (28, 106, 272, 411, 506, 577).

If one is interested either in reporting an isolated microorganism as having hydrocarbon-metabolizing abilities or in performing enumerations of hydrocarbon-degrading microorganisms, it is essential to include proper controls. Ample evidence is available to illustrate that non-hydrocarbon-degrading microorganisms will develop on agar plates prepared with solid, liquid, or volatile hydrocarbons due to the presence of utilizable carbon even in purified agarose (60, 504). In an evaluation of mineral agar plates with and without toluene-xylene fumes, it was revealed that little selection was provided against non-toluene- and non-xylene-degrading bacteria. Despite the caution to incubate plates with and without hydrocarbon, studies with oil agar to enumerate hydrocarbon-degrading bacteria without reporting proper controls can still be found. This type of report should be examined with care.

In an attempt to overcome the problem with trace carbon in agar preparations, some researchers turned to the use of silica gel as a solidifying agent. However, this tedious procedure has not enjoyed widespread use. If isolates are not required, a rapid MPN test (sheen-screen) with tissue culture plates can be employed for nonvolatile hydrocarbons based on the formation of emulsions, avoiding the problem of trace carbon contamination altogether (77). A similar assay to screen for hydrocarbon degraders based on a redox indicator has been described (236) and combined with the sheen-screen to produce an MPN assay based on both emulsification and respiration (633).

Numerous studies have attempted to describe microbe-microbe and microbe-hydrocarbon interactions by extrapolating from detailed laboratory studies with isolates from hydrocarbon-contaminated environments. For example, evaluations of functional and physiological isolate groupings have been carried out in an effort to quantify the oil emulsification abilities and type of hydrocarbon accession mode used by environmental isolates (67). Researchers have also constructed simplified consortia containing several well-defined strains in an effort to

identify specific processes that may be important in environmental settings.

In a recent study evaluating 10 strains enriched with phenanthrene as the sole carbon and energy source (7), isolates were examined without confounding interactions associated with complex media, substrates, and microbial mixtures. Strains from eight sites were able to metabolize PAHs with two to five rings following growth on phenanthrene. In terms of metabolism (oxidation, mineralization, or removal), each strain was unique with respect to substrate specificity, and all could oxidize at least one intermediate of the two known PAH degradation pathways (salicylate or phthalate). Despite widespread ability to metabolize benz[a]anthracene, chrysene, and benz[a]pyrene, none of the strains could mineralize pyrene alone. This led the authors to conclude that unique cometabolic processes are required for pyrene removal in natural environments. This is a common conclusion that, while probably correct, is typically unsubstantiated by any direct evidence or description of the specific processes involved.

Komukai-Nakamura et al. (340) evaluated various mixtures of an alkane-utilizing *Acinetobacter* spp. and a *Rhodococcus* sp., an alkylbenzene-degrading *Pseudomonas putida*, and a phenanthrene-utilizing *Sphingomonas* sp. in an attempt to elucidate how alkane- and aromatic-degrading microorganisms interact. The degradation of Arabian light crude oil was monitored, and a combination of the *Acinetobacter* sp. and *P. putida* was as effective as a mixture of the four microorganisms, degrading 40% of the saturates and 21% of the aromatics. Respirometry showed that *P. putida* was able to evolve CO<sub>2</sub> from unidentified metabolites of *n*-octylbenzene produced by the *Acinetobacter* sp. Many bioremediation companies offer such mixed cultures for sale to cope with environmental pollution (342), but third-party testing of such products has not proven them to be more effective than autochthonous microbial communities once additional nutrients and sorbents are removed (611, 638). Standard assay procedures with simple consortia are being developed for Environment Canada (199, 198) and the U.S. Environmental Protection Agency (232) in order to test such products.

These types of study are essential for understanding general mechanisms but do not reveal environmental importance. To achieve a greater understanding, the molecular biology and biochemistry of the processes need to be understood in detail so that gene expression can be correlated to activity. For example, using green fluorescent protein fusions, Holden et al. (258) showed that, in contrast to liquid cultures, expression of genes for rhamnolipid and PA bioemulsifying protein did not improve biodegradation of *n*-hexadecane in an unmixed sand culture. Instead, adherence to the hydrocarbon-water interface was more important for biodegradation.

Aside from isolating and identifying microorganisms present in hydrocarbon-impacted environments, descriptions of microbial communities have been based solely on functional characteristics. Normally based on MPN assays, dividing communities into physiological types is best served if numerous selective media are used and associated with relevant site characteristics. The MPN has appeared to be particularly useful for studying anaerobic systems, as it is sensitive, even when slow-growing anaerobes are being studied. Kämpfer et al. (301) monitored in situ bioremediation of a waste oil-contaminated

site subjected to various bioremediation treatments. Both groundwater and soil samples were taken with the aim of correlating microbiological and chemical data to assess bioremediation potential. Microorganisms were divided into the following classes: methylotrophic, facultative anaerobes, denitrifiers, sulfate reducers, oil-degrading denitrifiers, and anaerobic vacuum gas-oil degraders. In addition, 3,466 bacterial isolates (42.5% gram-positive) from R2A agar were identified, with >70% being previously reported as hydrocarbon degraders. While this is an impressive number of isolates, there is no indication of how important these isolates are in that particular environment. A separate study of a crude oil-contaminated aquifer (51) used a similar MPN approach to study ecological succession, microbial nutrient demands, and the importance of free-living versus attached populations. MPN determinations of aerobes, denitrifiers, iron reducers, heterotrophic fermenters, sulfate reducers, and methanogens were used. The dominant physiological types were consistent with the known geochemical evolution of the contaminant plume, from iron-reducing to methanogenic.

In Antarctica, Delille et al. (145) examined seasonal changes in the functional diversity of ice bacteria over 9 months in uncontaminated, contaminated, and treated (Inipol EAP22 fertilizer) plots. Total bacteria (acridine orange) saprophytes, and hydrocarbon-utilizing bacteria (MPN) were assayed. In all cases, changes in total bacterial abundance, reaching a minimum in the winter ( $<10^5$  cells ml<sup>-1</sup>), were correlated with seasonal variations. Following crude oil or diesel fuel contamination, bacterial counts increased, with increases in oil-degrading bacteria from 0.001% to 10%. Both saprophytic and oil-degrading bacteria increased with Inipol addition. In contrast, the underlying seawater showed limited variation between control and contaminated plots. In lieu of MPN assays, direct immunofluorescence and enzyme-linked immunosorbent assay have been used for nearly real-time quantification of hydrocarbon-degrading organisms (76). Immunodetection was shown to be applicable to complex sample matrices for rapid field evaluation. Antibody mixtures of sufficient specificity could potentially be developed to target specific microbial groups, although, in most situations, tracking the expression of specific genes involved in hydrocarbon metabolism would be of greater utility.

The most effective uses of an MPN approach, or indeed any approach to characterize a petroleum-impacted microbial community, has been realized when evaluating the role of a particular microbial group during remediation. For example, during enhanced oil recovery by water flooding, wells are often contaminated with hydrogen sulfide-producing sulfate-reducing bacteria that result in the souring of sweet crude oils. Biocides have often been found to be ineffective in controlling this problem, while nitrate addition has been used with some success (165, 166, 607). Eckford and Fedorak (165, 166) undertook an MPN-based study of some western Canadian oil field waters to show that nitrate addition stimulates the growth of heterotrophic nitrate-reducing bacteria that outcompete sulfate-reducing bacteria, presumably due to more favorable metabolic energetics. Nitrate-reducing bacteria have been neglected in the study of petroleum reservoirs (398), which illustrates that a circular approach to community studies, whereby

non-culture-based approaches lead to the development of new isolation techniques and vice versa, is recommended.

Total community analyses have been carried out with phospholipid fatty acid analysis profiles and Biolog substrate utilization patterns. In Australia, phospholipid fatty acid analysis profiles were evaluated as a method to provide insight into the monitoring-only approach during management of a gasoline-contaminated aquifer (202). Principal-component analysis did not reveal any clear groupings with respect to an aromatic hydrocarbon plume, and phospholipid fatty acid profiles were rejected as expensive and technically difficult for their purpose. A similar study (183) used total phospholipid fatty acid profiles to evaluate microbial community structure and biomass levels in a JP-4 jet fuel-contaminated aquifer. Aerobic and anaerobic zones were examined, and specific fatty acids were used in an attempt to draw conclusions with respect to the presence of aerobes and anaerobes, but overall, phospholipid fatty acid patterns are not sufficiently powerful to provide solid data about the presence of specific microorganisms in a community, let alone provide insight into their function.

Protein banding pattern analysis as a method to infer the function of isolates from a contaminated aquifer was found to suffer from the same limitations when evaluated by Ridgway et al. (513). A total of 297 isolates were screened for the ability to use gasoline vapor as a sole carbon and energy source and were pooled into 111 groups based on the usage pattern of 15 different volatile organic hydrocarbons. Following identification, sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns were used to regroup the isolates. Fifty-one groups were resolved that partitioned into two broad classes (metabolically diverse and metabolically restricted), but catabolic activity could not be predicted.

Berthe-Corti and Bruns (57) used Biolog substrate utilization patterns to evaluate the functional diversity of microbial communities in continuous-flowthrough cultures treating  $C_{16}$ -contaminated intertidal sediments. Standard dissolved oxygen and dilution rate effects typically used in situ remediations were implemented because it is desirable to determine if adaptations to low oxygen are due to changes in microbial community structure or metabolic adaptations of specific populations. Measurements of  $C_{16}$  degradation, product formation, oxygen consumption, total heterotrophs, and MPN determinations of nitrate reducers, sulfate reducers, and  $C_{16}$ -utilizing bacteria were combined with Biolog data. It was observed that substrate utilization became more limited, especially at low dissolved oxygen (0.4%) levels. Other parameters ( $C_{16}$  degradation, protein production, and oxygen consumption) increased with dilution independently of dissolved oxygen. Overall, the level of dissolved oxygen (80% or 0.4%) appeared to dictate the structure of the microbial community.

Lindstrom et al. (386) evaluated the long-term effects of a 1976 experimental sub-Arctic oil spill in Alaska by examining soil population structure and community-level metabolism. No differences in total bacterial numbers or soil carbon mineralization were detected, while hydrocarbon degraders (based on the sheen-screen assay) were elevated at the oil-contaminated site. Nitrogen mineralization and metabolically active microorganisms were abundant at the contaminated site. A kinetic analysis of the Biolog results was used to avoid problems with inoculum density and time-of-reading effects. Taken together,

the evidence was interpreted to conclude that the oil resulted in diminished microbial population diversity and selection for metabolic generalists even after extended exposure times. However, the importance of the observations in terms of overall ecosystem function is difficult to determine.

### Culture-Independent Approaches

At this time, we are beginning to understand the astonishing diversity of microbial populations and communities in the environment. Coming to grips with the inherent variability in microbial communities over space and time, even in the absence of petroleum hydrocarbons, remains a major challenge. Culture-independent approaches to microbial community analyses have recently enjoyed a surge in popularity as new techniques have been developed and are available in most major research institutions. Molecular descriptions of microbial communities now dominate the literature in all areas of microbial ecology, not just petroleum microbiology.

To be successful in the future, rapid automated systems will be required to process and evaluate vast quantities of data in order to subtract background variability. Even then, care must be taken to realize that, while molecular methods are powerful and attractive, the genetic composition of a community cannot be used to extrapolate ecosystem function. Kent and Triplett (317) summarized the current state of microbial community analysis succinctly: "The current era of investigation can be viewed as the descriptive phase, which is necessary prior to a testing phase where we will learn the role and perhaps the functional redundancy of the perhaps hundreds of millions of operational taxonomic units in soils on earth."

A few of the recent studies will be discussed here, and it is important to note that most studies involving culture-independent characterization of petroleum-impacted microbial communities have included other measures of microbial activity with culture-dependent methods. This is a requirement for making sense of data generated from culture-independent methods and to allow the development and evaluation of new methods.

Bulk measurements of total community DNA in a manner analogous to phospholipid fatty acid analysis and protein banding patterns have been used in an attempt to detect perturbations and changes in petroleum-impacted environments. Unlike phospholipid fatty acid analysis, specific microorganisms can be identified if the genetic material is extracted from each individual band following electrophoresis and then sequenced. This practice is time-consuming, and identification results, while intriguing, are often left without further attempts to isolate the observed organisms.

Shi et al. (560) used Domain probe analysis to examine community structure in pristine and fuel-contaminated aquifers. The predominantly bacterial populations were further divided (43 to 65%  $\beta$ - and  $\gamma$ -proteobacteria, 31 to 35%  $\alpha$ -proteobacteria, 15 to 18% sulfate-reducing bacteria, 5 to 10% high G+C). Physical-chemical data and the lack of members of the *Archaea* suggest that methanogenesis was not occurring in the aquifer. Øvreås et al. (468) used denaturing gradient gel electrophoresis (DGGE), sequencing, and DNA reassociation plots in combination with measurement of methane and methanol oxidation measurements to show a decrease in diversity

with a concomitant increase in known methanotrophs upon methane perturbation of agricultural soils.

MacNaughton et al. (394) used 16S rRNA PCR-DGGE and phospholipid fatty acid analysis to identify populations responsible for decontamination while evaluating oil spill bioremediation techniques and to help define an endpoint for substrate removal. Phospholipid fatty acid analysis, PCR-DGGE patterns, degradation rates, and hydrocarbon degraders (MPN) were similar for plots with nutrient and with nutrient plus inoculum. Complex banding patterns and low reproducibility were encountered, along with some disagreements between phospholipid fatty acid analysis and DGGE analysis. However, two novel bands, closely related to *Flexibacter-Cytophaga-Bacteroides* were detected in all nutrient-amended sites. Their contribution to enhanced degradation remains speculative. Rooney-Varga et al. (521) also used a mixed approach to evaluate anaerobic benzene degradation in a petroleum-contaminated aquifer. Phospholipid fatty acid analysis, MPN-PCR, and DGGE of 16S ribosomal DNA along with selective enrichment and biodegradation studies were used. Increased diversity at contaminated sites was observed along with higher phospholipid fatty acid contents. MPN-PCR indicated that *Geobacteriaceae* spp. were important at the site, which disagreed with phospholipid fatty acid profiles. This may be an indication that, while phospholipid fatty acid analysis can be useful for identifying isolated microorganisms, its utility as a tool for extrapolating the identity of individual community members from a total phospholipid fatty acid pattern is limited.

To date, community characterizations have been, for the most part, applied to field situations. Hydrocarbon-contaminated or impacted sites rather than fermentor-based treatment systems have been the target of characterization. Thus, this type of system may be useful for developing methods in a more controlled environment. Colores et al. (128) studied surfactant effects on C<sub>16</sub> and phenanthrene degradation by a mixed culture in laboratory microcosms by respirometry, 16S rRNA DGGE, and culture techniques. They found that surfactant levels close to the critical micellization in soil inhibited mineralization and shifted the community from *Rhodococcus* and *Nocardia* populations to *Pseudomonas* and *Alcaligenes* species able to degrade both surfactant and hydrocarbon. Of 60 isolates, 11 unique DGGE banding patterns were observed, three of which (*Rhodococcus*, *Pseudomonas*, and *Alcaligenes*) corresponded to major bands from the whole-community analysis.

It is apparent that total community approaches such as 16S rRNA DGGE banding patterns are not the end-all in understanding microbial communities or providing sufficient power to address specific hypotheses (565). More information is often available when gene probes for specific isolates, genotypes, or metabolic activities are used, and approaches to achieve this are being applied in both aerobic and anaerobic systems (117, 128, 156, 257, 304, 478, 514, 582, 609).

An excellent example of this has come out of Voordouw's laboratory at the University of Calgary. That group has published extensively on the use of molecular methods for the quantitative analysis of sulfate-reducing bacterial communities in oil fields (263, 605, 646). Sulfate-reducing bacteria play a key role in anaerobic corrosion in oil and gas fields, and elucidating their modes of action is important to oil companies. To this end, metabolic activity tests are useful but do not provide

information about specific species. Early work showed that gene probes based on the [Fe], [NiFe], and [NiFeS] hydrogenases could be used to identify *Desulfovibrio* spp. (648).

The observation that many specific hydrogenase probes failed to hybridize with sulfate-reducing bacterial isolates led to the development of reverse-sample genome probing (645). This technique allows the total DNA from a community to be quantitatively (649) analyzed in a single step. The proportion of the community being analyzed is related to the quantity of probe in the master filter, and a quantitative approach has been developed (649), and adding probes for non-sulfate-reducing bacteria to a filter is straightforward (647). Biofilm formation (649), nitrate injection (607), and diamine biocide (606) effects with respect to community composition and functional properties have been described. The approach has also been used for evaluating hydrocarbon-degrading bacteria in soil exposed to dicyclopentadiene (556), although it must be kept in mind that important groups of organisms may be missed with this method and that the presence of a specific microorganism does not indicate that it is active.

From a remedial perspective, tracking specific genes expected to be present in isolates from hydrocarbon-impacted environments may be more useful at this time, especially if workable methods for mRNA can be developed. Early work with gene probes following the *Exxon Valdez* spill revealed that bacterial populations containing both the *xylE* and *alkB* genes could be detected in environmental samples (578). In laboratory columns, proportions of *xylE* and *ndoB* (polycyclic aromatic hydrocarbon degradation) populations from an aquifer community were monitored during degradation of creosote-related PAHs (261). Isolates grown on tryptone-yeast extract medium were probed, and it was found that *p*-cresol addition resulted in a 100-fold increase in total culturable bacteria, with a threefold increase in *xylE*- and *ndoB*-positive populations. Langworthy et al. (359) found *nahA* and *alkB* in higher frequencies at PAH-contaminated sites, although these genes, along with *nahH* and *todC1/C2* were detected at pristine sites as well. Laurie and Lloyd-Jones (365) recently used competitive PCR to illustrate that the newly described *phn* genes of *Burkholderia* sp. strain RP007 may have greater ecological significance than *nah*-like genes for PAH degradation. The *phn* genes, while encoding the identical biodegradation pathway, have low sequence homology to *nah*, a different gene order, and are present in the organisms that are rarely cultured in the laboratory.

If the biochemistry and genetic diversity are known, gene probe suites have greater potential for accurately evaluating bacterial degradative potential (234, 424), although the application of a small number of probes may be effective if meaningful hypotheses are tested (565). Recent advances in characterizing alkane metabolism in a number of organisms have allowed the production of a variety of primers to detect, for example, the *alkB* gene from *P. putida* GPo1 (573). As more strains are tested and more probes are produced, it is becoming clear that, while different alkane hydroxylases can be found in phylogenetically distant microorganisms (19), many probes will only provide information on the presence of a similar gene in closely related strains. Thus, the usefulness of such gene probes will grow as the diversity of genes responsible for hy-

TABLE 4. Evaluation of various petroleum sludge treatment technologies

Remediation	Technology	Comments
Bioremediation	Bioreactor	Application of natural and specialized microorganisms in controlled environmental and nutritional conditions, high biodegradation rates, accommodates variety of sludges, nonhazardous residues, on-site operation, cost-effective
	Landfarming	Uses natural microbial population and supplements of mineral nutrients, slow degradation rates, year-round operation difficult, potential to contaminate ground and surface water, cost-effective
	Biopiling	Uses natural microbial population and supplemented nutrients and air, slow degradation rates, year-round operation difficult, potential to contaminate ground and surface water
	Bioventing	A combination of advective soil venting and biodegradation method for in situ treatment of soils, most of the lighter hydrocarbons are volatilized
	Biostimulation/bioaugmentation	Application of mineral nutrients/surfactants and/or microorganisms to stimulate or supplement natural microbial population at contaminated site
	Phytoremediation	Uses plants and rhizospheric microorganisms for the treatment of contaminated soil, potential for removal of petroleum contaminants being evaluated, presumably cost-effective
Physicochemical	Incineration	High-temperature treatment, air pollution risks, expensive control equipment, high capital cost
	Thermal desorption	High-temperature oil removal and recovery method from oily solids, high capital and material preparation costs, nonhazardous residues
	Coker	Complicated sludge preparation for coker feed, some oil recovery, high capital and transportation costs
	Cement kiln	Complicated sludge preparation for use of fuel, high material preparation, transportation, and disposal costs
	Solvent extraction	Uses solvents and centrifugation or filtration for the separation of oil from sludges, safety hazard with solvent use, high capital cost

drocarbon metabolism is better appreciated (120, 573, 644, 661, 662, 663).

This field will be greatly advanced if genome projects are initiated to sequence environmentally important microorganisms, including fungi, if the diversity of hydrocarbon metabolic pathways is better characterized, and if tools to monitor gene expression on a large scale are developed (146). Finally, the most important point to recall when embarking on a community-based study is that a clear, testable hypothesis be framed at the outset.

#### MICROBIAL TREATMENT OF PETROLEUM WASTE

Earlier reviews on hydrocarbon biodegradation have described bioremediation efforts, including the use of chemicals (surfactants and dispersants) (126, 194, 370, 489, 522, 599). The general importance of relying on the indigenous microbial population, which presumably resists tidal washing by association with oily surfaces rather than on inocula, has been emphasized.

Environmental impacts from the petroleum industry derive from recovery, transport, refining, and product usage. Only 10% of the last is attributed to high-profile marine oil spill catastrophes resulting in shoreline contamination (36, 489). In various operations of production, processing, and storage, large volumes of waste are generated as oily sludges (404). Hydrocarbons bind strongly to solid surfaces, including soils, and remediation of these materials represents a significant challenge. The lighter and often toxic hydrocarbon compo-

nents tend to volatilize into the atmosphere, reducing air quality and threatening human and animal health. High levels of sulfur compounds are also emitted in petrochemical waste streams, which require treatment. The following sections will focus on treatment of petroleum-contaminated solids, biofiltration of volatile compounds from air streams, and removal of sulfur compounds from waste streams. Hence, in contrast to earlier reviews which focused on clean-up of contaminated sites, the main emphasis here is on bioprocessing of waste streams.

#### Treatment of Contaminated Soils and Sludges

Compared to physicochemical methods, bioremediation offers an effective technology for the treatment of oil pollution because the majority of molecules in the crude oil and refined products are biodegradable and oil-degrading microorganisms are ubiquitous (Table 4) (6, 107, 185). However, abiotic losses due to evaporation, dispersion, and photooxidation also play a major role in decontamination of oil spill environments (211, 535). In the case of in situ subsurface bioremediation processes, the greatest challenges relate to engineering of the subsurface environment so that microbes can thrive there and effectively degrade the contaminants present. Biological methods for processing of oily sludges and oil-contaminated soils in landfarming, biopiling/composting, bioventing, and bioreactor configurations have been well documented (30, 342, 489, 652).

**Factors affecting bioremediation.** The rate of microbial degradation of crude oil or oil waste depends on a variety of

factors, including the physical conditions and the nature, concentration, and ratios of various structural classes of hydrocarbons present, the bioavailability of the substrate, and the properties of the biological system involved (337, 593, 637, 669, 684). A generalized sequence of petroleum components in order of decreasing biodegradability is represented as follows (268): *n*-alkanes > branched-chain alkanes > branched alkenes > low-molecular-weight *n*-alkyl aromatics > monoaromatics > cyclic alkanes > polynuclear aromatics >> asphaltene. Predictive models for estimating the extent of petroleum hydrocarbon biodegradation (268) and diffusion-controlled bioavailability of crude oil components (621) have been developed. Properly chosen chemical surfactants may enhance biodegradation (79, 80, 453, 529, 634). The efficiency of processes for degradation of hydrocarbons will also depend on the nature of the hydrocarbon-contaminated material, the environmental conditions, and the characteristics of the microbial population that is present.

Assuming that microbes are present, nutrient availability, especially of nitrogen and phosphorus, appears to be the most common limiting factor (494, 526). Laboratory and field experiments with inorganic nitrogen and phosphate fertilizers and organic fertilizers, including fish bones, fish or animal meal, biosurfactants, and bulking agents, have shown success (68, 241, 371, 372, 428, 446, 458, 512, 635, 640).

Strategies for microbial degradation of petroleum contaminants or wastes manifest themselves in processes having different degrees of complexity and technological requirements. Bioremediation of contaminants in soil by natural attenuation requires no human intervention, whereas implementation of accelerated and controlled bioreactor-based processes may be directed to exploiting microbial technology and bioprocess engineering to optimize the rates and extents of contaminant degradation.

In simple bioremediation systems, which require little or no microbiological expertise, process-limiting factors often relate to nutrient or oxygen availability or the lack of relatively homogeneous conditions throughout the contaminated medium. Microbial growth and degradation processes operating under such conditions are typically variable and suboptimal, leading at best to prolonged degradation cycles (443). Processes are often unreliable, and required contaminant degradation endpoints are often not achieved throughout the medium. These processes tend to ignore the realities of enzyme and cell substrate saturation kinetics, where rates of degradation slow as contaminant concentrations fall, with resulting reductions in the viable microbial population. When contaminants are degraded by cometabolism, early elimination of the cosubstrates, necessary for degradation of these contaminants, can halt the degradation processes. The nonhomogeneous and unpredictable nature of these processes makes them intensive in terms of sampling and analytical activities, as patterns of contaminant removal have to be monitored throughout a three-dimensional grid.

The need for intensive monitoring represents a major justification for the implementation of more optimized biodegradation processes, which ensure contaminants are efficiently biodegraded to defined criteria. Short-term real estate development plans or measures to afford greater protection to the environment or to comply with increasingly stringent environ-

TABLE 5. Major biosurfactants produced by microorganisms<sup>a</sup>

Class	Biosurfactant	Microorganisms
Low molecular weight	Rhamnolipids	<i>Pseudomonas aeruginosa</i>
	Trehalose lipids	<i>Arthrobacter paraffineus</i> <i>Rhodococcus erythropolis</i> <i>Mycobacterium</i> spp. <i>Candida lipolytica</i>
	Sophorose lipids	<i>Torulopsis bombicola</i>
	Viscosin	<i>Pseudomonas fluorescens</i>
	Surfactin	<i>Bacillus subtilis</i>
	Polymixins	<i>Bacillus polymyxa</i>
	Gramicidin S	<i>Bacillus brevis</i>
	Phospholipids	<i>Acinetobacter</i> spp. <i>Thiobacillus thiooxidans</i>
	Lipopeptides	<i>Bacillus pumilus</i> <i>Bacillus licheniformis</i> <i>Pseudomonas fluorescens</i>
	Polyol lipids	<i>Rhodotorula glutinis</i> <i>Rhodotorula graminis</i>
	Serrawettin	<i>Serratia marcescens</i>
	Fatty acids (corynomycolic acids, spiculisporic acids)	<i>Corynebacterium lepus</i> <i>Arthrobacter paraffineus</i> <i>Penicillium spiculispornum</i> <i>Talaromyces trachyspermus</i>
	Sulfonylipids	<i>Capnocytophaga</i> spp.
	Diglycosyl diglycerides	<i>Lactobacillus fermentii</i>
High molecular weight	Alasan	<i>Acinetobacter radioresistens</i>
	Emulsan	<i>Acinetobacter calcoaceticus</i>
	Biodispersan	<i>Acinetobacter calcoaceticus</i>
	Liposan	<i>Candida lipolytica</i>
	Mannan-lipoprotein	<i>Candida tropicalis</i>
	Food emulsifier	<i>Candida utilis</i>
	Insecticide emulsifier	<i>Pseudomonas tralucida</i>
	Sulfated polysaccharide	<i>Halomonas eurihalina</i>
	Acetyl heteropolysaccharide	<i>Sphingomonas paucimobilis</i>

<sup>a</sup> Data are from references 41, 42, 97, 149, 400, and 401.

mental regulations require accelerated remediation of contaminated sites. Increasing levels of microbial expertise may be exploited in processes for accelerated transformation of petroleum contaminants and wastes.

Several laboratory and field investigations have indicated that the addition of commercial microbial cultures (bioaugmentation) (118, 340, 431, 637) did not significantly enhance rates of oil biodegradation over that achieved by nutrient enrichment (biostimulation) of the natural microbial population (186, 494, 639). The *Exxon Valdez* bioremediation experience, in particular, has been viewed by many as a general rule that bioaugmentation is ineffective in petroleum and other biodegradation processes. This begs two questions: Is there ever a role for inocula in petroleum degradation processes? Is there any potential to exploit recombinant organisms in the practice of environmental bioremediation and waste treatment?

The low water solubilities of the majority of petroleum hydrocarbon compounds have the potential to limit the capacity of microbes, which generally exist in aqueous phases, to access and degrade these substrates. Hydrocarbon-degrading microbes produce a variety of biosurfactants (Table 5) as part of



their cell surface or as molecules released extracellularly (43, 86, 87, 88, 191, 401, 450, 451, 524, 527, 541, 567). These biosurfactants and added chemical surfactants enhance removal of petroleum hydrocarbons from soil or solid surfaces. However, both enhancement and inhibition of biodegradation of hydrocarbons have been observed (35, 356, 618). Suppression of their production, by use of inhibitors or mutagens, retards the ability of these bacteria to degrade oil (41, 491). The low-molecular-weight biosurfactants (glycolipids, lipopeptides) are more effective in lowering the interfacial and surface tensions, whereas the high-molecular-weight biosurfactants (amphipathic polysaccharides, proteins, lipopolysaccharides, and lipoproteins) are effective stabilizers of oil-in-water emulsions (41, 97, 149, 384, 401, 525).

Many studies have characterized the roles of biosurfactants in biodegradation by observing the effects of fractionated preparations (42, 121, 178, 182, 254, 282, 200, 306, 456, 524, 525, 629, 688, 689). However, the successful application of biosurfactants in bioremediation of petroleum pollutants will require precise targeting to the physical and chemical nature of the pollutant-affecting areas. Although many laboratory studies indicate the potential for use of biosurfactants in field conditions, a lot remains to be demonstrated in cost-effective treatment of marine oil spills and petroleum-contaminated soils compared to chemical surfactants.

Chemical surfactants have the ability to emulsify or pseudosolubilize poorly water-soluble compounds thus potentially improving their accessibility to microorganisms. Properties of chemical surfactants that influence their efficacy include charge (nonionic, anionic or cationic), hydrophilic-lipophilic balance (a measure of surfactant lipophilicity), and critical micellar concentration (the concentration at which surface tension reaches a minimum and surfactant monomers aggregate into micelles). Surfactants with hydrophilic-lipophilic balance values from 3 to 6 and 8 to 15 generally promote formation of water-in-oil and oil-in-water emulsions, respectively. Biodegradation of certain poorly soluble petroleum hydrocarbons may be inhibited by surfactants as a result of (i) toxicity by high concentration of surfactant or soluble hydrocarbon; (ii) preferential metabolism of the surfactant itself; (iii) interference with the membrane uptake process; or (iv) reduced bioavailability of micellar hydrocarbons (167, 446, 529).

Typical surfactant concentrations required to wash contaminants out of soil are 1 to 2%, whereas the same contaminants may be solubilized in an aqueous solution at a surfactant concentration of 0.1 to 0.2%. Much of the surfactant added to soil is ineffective as it becomes sorbed to soil particles. Micellarization of the contaminant (at or above the surfactant critical micellar concentration) may prevent access to the contaminant by the microorganism. Diluting the contaminated medium to get the surfactant concentration below its critical micellar concentration can facilitate microbial access and contaminant degradation (59). When the effects of surfactant physicochemical properties (hydrophilic-lipophilic balance and molecular structure) on the biodegradation of crude oil by a mixed bacterial culture were examined, hydrophilic-lipophilic balance-13 nonylphenoethoxylate substantially enhanced biodegradation at surfactant concentrations of more than critical micellar concentration value (634). Surfactants from other chemical classes

with hydrophilic-lipophilic balance values of 13 had no effect or were inhibitory.

The range of stimulatory and inhibitory effects of surfactants on hydrocarbon degradation reported in the literature may not be contradictory but simply describe unique cases based on the nature of the hydrocarbon contaminants, characteristics of the contaminated medium, surfactant properties and the physiology of the organisms involved (260, 631). Understanding how these four elements interact may enable us to design surfactant-enhanced bioremediation systems on a more rational basis (36, 342, 367, 630).

In the following section, the variety of petroleum biodegradation processes will be reviewed, starting with the processes requiring the least microbial expertise and moving on to processes with increasing levels of microbial technological complexity.

**Passive bioremediation processes.** Natural attenuation, the least invasive approach to bioremediation, requires no intervention other than to demonstrate the progress of the degradation mediated by the indigenous microbial population, and its efficacy remains controversial (270).

Plants and their rhizospheric microorganisms (phytoremediation) can participate in hydrocarbon remediation (47, 151, 162, 262, 402, 419, 422, 498, 536, 549, 595, 650, 678). Plant root exudates can supply carbon and nitrogen sources for microbial growth (12, 486), raising the densities of rhizospheric bacteria by orders of magnitude more than the population in the surrounding soil (12, 138, 536), and enzymes may be produced that degrade organic contaminants (69, 393, 550). Phytoremediation is not a suitable method for remediation of high-volume oily wastes. Volatile organic carbons can be taken up by plants and transpired to the atmosphere without transformation in a process known as phytovolatilization, which is not an acceptable environmental solution. There is limited plant uptake of more hydrophobic and larger petroleum components.

Wetland use in the petroleum industry for removal of inorganic and organic contaminants and toxicity from hydrocarbon wastes was reviewed by Knight et al. (336). Contaminant removal effectiveness depended more on hydraulic loading and influent concentrations than on internal plant communities and water depth. Often biodegradation is accompanied by other removal mechanisms (535). Aerobic processes generally predominate, and the toxicity of contaminants or metabolites is often a problem. The availability of fertilizer and oxygen is often rate limiting (240, 264, 383, 442, 561).

In general, therefore, these more passive remediation approaches are unlikely to provide sufficient capacity for remediation of high-volume petroleum wastes with their relatively concentrated hydrocarbon contaminant level (typically 2 to 20%).

**Landfarming of oily wastes.** While landfarming of refinery and wellhead oily sludges is no longer considered environmentally acceptable, it is still being used as an oily sludge treatment and disposal method in many parts of the world (29, 44, 267). As a starting point, large uncontaminated tracts of land are first deliberately contaminated, followed by bioremediation of the less recalcitrant oil fractions. Large refineries, having capacities of 200,000 to 500,000 barrels per day can produce as much as 10,000 cubic meters of sludge per annum. These landfarming operations can therefore result in tying up large

areas of land which will later have to be decommissioned when more environmentally desirable processes are implemented.

Large quantities of volatile organic carbons present in these wastes, which are hazardous to health and which cause tropospheric ozone production, are typically transferred to the atmosphere rather than biodegraded, facilitated by spraying the waste on the land and then routinely tilling the soil to promote gas transfer. In the *Exxon Valdez* spill in the relatively cold Alaskan climate, 15 to 20% of the oil was reported to be lost to the atmosphere by volatilization (219).

Lack of control over the parameters affecting microbial activity (temperature, pH, moisture, aeration, mixing, and circulation) prolongs treatment time (62, 177, 267, 269, 366, 389, 405, 406, 407, 432). Maximum contaminant degradation occurs in the tilled surface, typically amounting to 10 to 20 cm of depth, although deeper aeration and mixing with ploughing and rotovating equipment has also been effectively implemented. The following examples indicate that typical degradation rates of 0.5 to 1% total petroleum hydrocarbon contents per month may be achieved with landfarming. (i) When refinery soil contaminated with 1.3% oil was treated with nutrients, surfactants, and microbial inoculants and the soil was regularly mixed and aerated with deep tilling equipment at air temperatures of around 25°C, total petroleum hydrocarbon contents were reduced by about 90% in 34 days (170). (ii) Landfarming of soil contaminated with 6% No. 6 fuel oil, with nutrient application, control of moisture, and aeration by ploughing and rotovating, resulted in an 80 to 90% reduction in total petroleum hydrocarbon contents in a 6-month span (196). (iii) Landfarming of kerosene-contaminated soil, depth up to 45 cm, with nutrient application and periodic tilling reduced contaminants from 8,700 ppm to 30 to 3,000 ppm (depending on soil depth) (270). Oxygen availability appeared to be a limitation in this project. (iv) Bosert et al. (65) characterized the fate of hydrocarbons during a laboratory study of oily sludge application to soil, simulating an active petrochemical plant landfarming operation. During intensive landfarming of petroleum waste, a gradual accumulation of petroleum hydrocarbons occurred in the soil over time, amounting to 13.8%, wt/wt. Of the total PAHs applied to the soil in the waste, the percentages remaining at the end of treatment were 1.4, 47.4, 78.5, and 78.3% for the 3-, 4-, 5-, and 6-ringed PAHs, respectively. Residual soil concentrations for pyrene and benzo[a]pyrene were 245 and 28 ppm, respectively, representing extents of degradation of 14.4 and 44.4%, respectively. At the end of the treatment period, 53% (155 mg of hydrocarbons per g of soil) of the applied hydrocarbons were removed from the soil, representing a degradation rate of 1%, wt/wt (hydrocarbons/soil) per 2 months.

Because of the trend to ban landfarming of petroleum sludges (175) and because they are considered hazardous wastes, oil companies are seeking other disposal solutions.

**Bioreactor-based processes.** Most of the rate-limiting and variability factors observed in landfarming of oily sludges may be eliminated in employing simple bioreactors where optimal performance can be achieved by controlling factors affecting rates and extents of microbial growth and oil transformation (270). Bioreactors can accommodate solids concentrations of 5 to 50% wt/vol. Through break up solid aggregates and dispersion of insoluble substrates, hydrocarbon desorption and con-

tact with the aqueous phase is promoted, resulting in increased biodegradation (119).

Bioreactor-based petroleum sludge degradation processes also allow management of volatile organic carbons. By creating reactor conditions which accelerate the process of bioremediation of volatile organic carbons, the biodegradation process rather than volatilization becomes the dominant volatile organic carbon removal mechanism (388, 632). Retaining the more volatile components, which are generally more biodegradable and more supportive of microbial growth and cell energy, supports degradation of the less volatile components, which may rely on cometabolic processes. In more prolonged hydrocarbon biodegradation processes, for example, landfarming, where volatile materials are lost to the atmosphere, the development of microbes on these substrates, containing the catabolic enzymes with relaxed substrate specificities to transform the more recalcitrant compounds, is not facilitated. Diesel fuel stimulated cometabolic mineralization of benzo[a]pyrene in culture and in soil (305, 302). The volatile components also help solubilize the more recalcitrant molecules, making them more bioavailable. The ability of paraffin oil to promote mineralization of pyrene was attributed to its solubilizing action (290).

Examples 1 to 3 below describe bioreactor processes having reactor cycle durations of 1 to 4 months (132, 466). Based on an assumed average total petroleum hydrocarbon contents content of 10% in these processes, average degradation rates ranged from about 0.1% to 0.3% total petroleum hydrocarbon contents per day. Example 1: French Limited, Crosby, Tex., refinery and petrochemical wastes were degraded in a slurry-phase aerated and mixed system (173, 174). The inoculum was indigenous microflora, and a novel mixing/aeration system (the MixFlo system) with pure oxygen rather than air was incorporated. Three hundred thousand tons of tar-like material was remediated in 11 months, with 85% of sludge contaminants being destroyed in 122 days. Example 2: Gulf Coast Refinery, a 1-million-gallon bioreactor was used to treat petroleum-impounded sludges (132). The inoculum was hydrocarbon-degrading organisms from a refinery wastewater activated sludge system. Aeration/mixing was done with float-mounted mixer/aerators. Other operating parameters were an average temperature of 22.6°C and nominal solids contents in the reactor of about 10%. A 50% reduction in oil and grease was obtained in 80 to 90 days. The extent of removal of PAHs was 90%. Example 3: Sugar Creek, Mo., refinery sludge treatment in a 5-million-gallon unlined reactor. The inoculum was activated sludge and prepared hydrocarbon cultures. A float-mounted aeration and mixing system was used. Oil and grease concentrations were reduced by 66% (<60 to 90 days), after which the solids were land applied to reduce residual PAHs to below 160 mg/kg (15).

Example 4: the petrozyme process utilizes a well-acclimated culture (see below), an optimized nutrient formulation, a surfactant, and implementing the process in an optimal temperature and pH range, a highly efficient petroleum sludge degradation process was shown to operate in a much shorter cycle time (571, 654). This process, employing eight bioreactors with a total capacity of 1.2 million liters, has been successfully operated for treatment of sludges produced from about 75% of Venezuela's refining capacity. The process has also been im-

plemented at a small number of refineries in the United States, Canada, and Mexico and typically degrades sludges having total petroleum hydrocarbon contents (total petroleum hydrocarbon contents) of 10% wt/vol. Average degradation rates were close to 1% of total petroleum hydrocarbon contents per day. For the initial batch, a mixed microbial culture, acclimated by weekly subculture on crude oil, was used as the inoculum. For each subsequent batch cycle, inoculation is achieved by carryover of a culture fraction from the previous batch. A sparged air-lift aeration system with no mechanical mixing was used. Nutrients and surfactant were formulated to maximize hydrocarbon accession to the microorganisms, microbial growth rates, and rates and extents of hydrocarbon degradation. The operating temperature (28 to 32°C) is maintained without temperature control in the stable Venezuelan climate. pH is maintained in the range from 6.4 to 7.6. The residence time was 10 to 12 days; the extent of degradation of total petroleum hydrocarbon contents was 97 to 99%; and residual PAHs comply with Environmental Protection Agency nonhazardous toxicity characteristics leaching procedure criteria. The process has operated consistently over hundreds of runs at pilot and full scale.

Recent progress in microbiology, molecular biology, and genetics is providing the driving force toward engineering improved biocatalysts (microbes and enzymes) for bioremediation (113, 483, 612). It also offers new tools to collect information on microbial populations in contaminated sites to aid in the evaluation and formulation of strategies for effective bioremediation (655). Risk-based regulatory approaches have continued to put stricter regulation on the field applications of genetically engineered microorganisms (154, 425).

The first demonstration of field release of a genetically engineered microorganism for bioremediation purposes involved use of the engineered strain *Pseudomonas fluorescens* HK44, containing naphthalene catabolic plasmid pUTK21 and a transposon-based bioluminescence-producing *lux* gene fused within a promoter for the naphthalene catabolic genes (515, 544). The environmental release occurred in six lysimeter structures containing soil with and without contaminant PAHs. Soil PAH concentrations were heterogeneously dispersed, spatially ranging from 0.04 to 192 ppm. Consequently, a precise evaluation of the effectiveness of *P. fluorescens* could not be adequately determined. However, the concept of developing a genetically engineered strain with the broad metabolic potential required to deal with the diverse array of hydrocarbon components of crude oil has serious shortcomings because of the range of new genes which would need to be incorporated. Moreover, the burden of maintaining all of these genes is likely to make the engineered strains noncompetitive in the natural environment (377).

The above examples describe the performance of microbial processes for degradation of waste hydrocarbons with different levels of process control and optimization. Typical degradation rates of 0.5 to 1% of total petroleum hydrocarbon contents/month obtained in landfarms can be increased to 0.1 to 0.3% per day in simple aerated bioreactor-based processes. With further control, optimization of media and culture conditions, including use of surfactants, degradation rates of 1% per day can be achieved. More-contained bioreactor-based systems also facilitate volatile organic carbon retention. Extents of deg-

radation in landfarming operations are poor, and very limited degradation of higher-molecular-weight PAHs was observed. In contrast, in optimized bioreactor biodegradation systems, overall degradation extents are very high, with reduction of PAHs to below nonhazardous criteria.

The diversity of metabolic pathways required to degrade the range of components in crude oil wastes is likely best provided by a mixed culture suitably acclimated on this substrate. It is unlikely that genetically engineered organisms can contribute to improving the best processes described above, even allowing for the remote possibility that such engineered strains could be used without taking prohibitive cost containment measures, because of the additional costs associated with maintaining a sterile environment excluding competing strains. Further expenses may be incurred if there are restrictions on release of the recombinant strain into the environment.

### Biofiltration of Volatile Organic Compounds

Two general types of biofilters exist: solid phase-gas phase biofilters and liquid phase-gas phase biofilters. The biofilter must be amended with appropriate nitrogen, phosphorus, and other nutrients. Inoculation may be used to shorten the start-up or acclimation period (3, 209, 155, 403, 296, 636, 691).

A recurring theme in processes discussed above is that large volumes of volatile components are often transferred to the atmosphere rather than being biodegraded. Petroleum-originating volatile organic carbons, especially BTEX compounds (benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene, and *p*-xylene), are classified as hazardous environmental priority pollutants. A number of well-established physical separation or destruction technologies exist for controlling the air emission of volatile organic carbons. However, stricter environmental regulations, high costs, and low public acceptance are driving the quest for dependable cost-effective methods for volatile organic carbon treatment, and biological methods are accepted as the most cost competitive.

Biological oxidation of volatile organic carbon vapors by microorganisms immobilized on a solid support material as biofilms and placed in reactors called biofilters provides an effective and inexpensive alternative for removal of volatile organic carbons (40, 134, 376). In these systems, the volatile organic carbon-containing gas phase passes through the high surface solid support phase containing microbial films with little resistance. The media sorb contaminants from the vapor stream and may supply organic and/or inorganic compounds for microbial growth and metabolism (140, 392).

In liquid-gas phase biofilters, the volatile organic carbon-contaminated gas may be sparged or bubbled through a liquid phase. A silicone membrane bioreactor system allowed rapid diffusion of volatile organic carbons and oxygen for the biodegradation of BTEX vapors (34). The system removed BTEX at rates of up to 30  $\mu\text{g h}^{-1} \text{cm}^{-2}$  of membrane area, with removal efficiencies ranging from 75% to 99% depending on the BTEX concentration and vapor flow rate. Other approaches achieved high gas transfer through use of fine bubble diffusers.

In order to keep the size of the biofilter in realistic proportion, contact or retention time for the gas stream in the biofilter has to be on the order of 30 to 90 s while achieving high

volatile organic carbon removal rates (typically 90%). Consequently, the biofilter must provide the conditions capable of maintaining a microbial population which can support these ambitious objectives. Biofilter microbial activity needs to be able to operate at gas flow rates of around 1 to 2 liters of gas per liter of biofilter capacity per min and degrade around 1 to 2 kg of volatile organic carbons per 1,000 liters of biofilter capacity per day (0.1 to 0.2% per day), which is only a little less than the performance quoted for optimized accelerated petroleum waste bioreactors (676).

Volatile organic carbon biofilters have to be very efficient high-density microbial systems capable of high rates of volatile organic carbon transformation. Optimized gas transfer from the mobile gas phase must be promoted by maximizing the surface area of the solid-phase biofilm or the gas-liquid interfacial area, where the stationary phases are solid and liquid, respectively. Mass transfer of the volatile organic carbons to the degrading microorganisms is particularly challenging because of their hydrophobic nature. Surfactants may be used to promote solubilization of the volatile organic carbons in the aqueous medium or at the solid surface and to increase transfer of the volatile organic carbons from the mobile gas phase (307).

#### Removal of $H_2S$ and $SO_x$

High quantities of  $H_2S$  and sulfoxides ( $SO_x$ ) produced in various petrochemical gas and liquid waste streams require treatment, and bacterial processes which purify these streams and convert these by-products to elemental sulfur are now being commercialized. The Thiopaq process ( $H_2S + [1/2] O_2 \rightarrow S^0 + H_2O$ ) is a desulfurization process for the production of elemental sulfur from  $H_2S$ -containing gas streams by sulfur-oxidizing bacteria (24, 471). Gas streams are first scrubbed with an aqueous washing liquid, with dissolution of the sulfur components into an aqueous phase ( $H_2S + OH \rightarrow HS^- + H_2O$ ). Sulfide-oxidizing thiobacilli, *Thiocalovibrio* and *Thioalcalobacteria* species, convert the sulfides to elemental sulfur ( $HS^- + [1/2] O_2 \rightarrow S^0 + OH^-$ ) in the presence of an electron acceptor at neutral pH (85, 284). The bacteria deposit the elemental sulfur outside the cell. The sulfur is separated in a sulfur separator, and the percolation water is recycled to the scrubber. pH- and redox-controlled bioreactors convert as much as 96% of the  $H_2S$ , which may be recovered as elemental sulfur and can be removed by available separation methods (620).

These processes with well-known sulfur-oxidizing strains and pathways have only recently been introduced. Environmental parameters are controlled to maximize the long-term process implementation. Technical and commercial efficacy will be determined in the coming years.

Sulfate-rich seawater, commonly injected into the oil reservoirs to enhance secondary oil recovery, may stimulate the growth of sulfate-reducing bacteria in the reservoirs, with subsequent  $H_2S$  production. This biogenic  $H_2S$  production, also known as reservoir souring, is of major concern to the oil industry.  $H_2S$  is corrosive, increases sulfur content in oil and gas, and may also lead to reservoir plugging (250). Reduction in  $H_2S$  formation by addition of nitrate to the injection water has been reported (508, 607). The beneficial effect of nitrate injection for stimulation of a competing group of nitrate-re-

ducing bacteria has been demonstrated in several model experiments (447, 508) and successful field applications (288, 607). Following nitrate injection, nitrite inhibition of sulfate-reducing bacteria and sulfide oxidation by nitrate-reducing bacteria have been suggested as the mechanisms for  $H_2S$  elimination (288, 447). Nitrite reductase-containing sulfate-reducing bacteria can overcome this inhibition by further reducing nitrite to ammonia (225). Nitrite reductase can be regarded as a resistance factor that prevents the inhibition of dissimilatory sulfate reduction by nitrite.

### MICROBIAL PROCESSES FOR RECOVERING AND UPGRADING PETROLEUM

#### Microbial Enhanced Oil Recovery

In microbial enhanced oil recovery processes, microbial technology is exploited in oil reservoirs to improve recovery (41, 122, 589). From a microbiologist's perspective, microbial enhanced oil recovery processes are somewhat akin to in situ bioremediation processes. Injected nutrients, together with indigenous or added microbes, promote in situ microbial growth and/or generation of products which mobilize additional oil and move it to producing wells through reservoir repressurization, interfacial tension/oil viscosity reduction, and selective plugging of the most permeable zones (81, 82). Alternatively, the oil-mobilizing microbial products may be produced by fermentation and injected into the reservoir.

This technology requires consideration of the physicochemical properties of the reservoir in terms of salinity, pH, temperature, pressure, and nutrient availability (319, 320). Only bacteria are considered promising candidates for microbial enhanced oil recovery. Molds, yeasts, algae, and protozoa are not suitable due to their size or inability to grow under the conditions present in reservoirs. Many petroleum reservoirs have high NaCl concentrations (286) and require the use of bacteria which can tolerate these conditions (558). Bacteria producing biosurfactants and polymers can grow at NaCl concentrations of up to 8% and selectively plug sandstone to create a biowall to recover additional oil (499).

One microbial enhanced oil recovery approach successively limits the carbon sources and increases the temperature, pressure, and salinity of the media to select microbial strains capable of growing on crude oil at 70 to 90°C, 2,000 to 2,500 lb/in<sup>2</sup>, and a salinity range of 1.3 to 2.5% (27). Thermophilic isolates potentially useful for microbial enhanced oil recovery have been described (14, 395). Extremely thermophilic anaerobes that grow at 80 to 110°C have been isolated and cultured in the laboratory. All of these organisms belonged to the archaeobacteria, living autotrophically on sulfur, hydrogen, and carbon dioxide by methanogenesis and heterotrophically on organic substrates by sulfur respiration or anaerobic fermentation.

A one-dimensional model was developed to simulate the microbial enhanced oil recovery process (150). The model involved five components (oil, bacteria, water, nutrients, and metabolites), with adsorption, diffusion, chemotaxis, growth and decay of bacteria, nutrient consumption, permeability damage, and porosity reduction effects. Comparison between the experimental and simulated results emphasized the validity

TABLE 6. Microbial products and their applications in enhanced oil recovery<sup>a</sup>

Product	Microorganism	Application in oil recovery
Biomass	<i>Bacillus licheniformis</i> <i>Leuconostoc mesenteroides</i> <i>Xanthomonas campestris</i>	Selective biomass plugging Viscosity reduction Oil degradation, wettability alteration
Biosurfactants (emulsan, sophorolipids, peptidolipid, rhamnolipid)	<i>Acinetobacter calcoaceticus</i> <i>Arthrobacter paraffineus</i> <i>Bacillus licheniformis</i> <i>Clostridium pasteurianum</i> <i>Corynebacterium fasciens</i> <i>Pseudomonas rubescens</i>	Emulsification, decrease of interfacial tension, viscosity reduction
Biopolymers (alginate, xanthan, dextran, pullulan)	<i>Bacillus polymyxa</i> <i>Brevibacterium viscogenes</i> <i>Leuconostoc mesenteroides</i> <i>Xanthomonas campestris</i>	Injectivity profile modification, mobility control
Solvents ( <i>n</i> -butanol, acetone, ethanol)	<i>Clostridium acetobutylicum</i> <i>Clostridium pasteurianum</i> <i>Zymomonas mobilis</i>	Oil dissolution, viscosity reduction
Acids (acetate, butyrate)	<i>Clostridium</i> spp. <i>Enterobacter aerogenes</i>	Permeability increase, emulsification
Gases (CO <sub>2</sub> , CH <sub>4</sub> , H <sub>2</sub> )	<i>Clostridium acetobutylicum</i> <i>Clostridium acetobutylicum</i> <i>Enterobacter aerogenes</i> <i>Methanobacterium</i> sp.	Increased pressure, oil swelling, decrease of interfacial tension, viscosity reduction, permeability increase

<sup>a</sup> Data are from references 41, 195, 499, 558, and 584.

of the simulator developed and determined its degree of accuracy (average absolute relative error, 8.323%). Oil recovery was found to be sensitive to variations in the concentration of injected bacteria, the size of the bacterial culture plug, incubation time, and residual oil saturation.

Microbial enhanced oil recovery-participating microorganisms produce a variety of fermentation products, e.g., carbon dioxide, methane, hydrogen, biosurfactants, and polysaccharides from crude oil, pure hydrocarbons, and a variety of non-hydrocarbon substrates (Table 6). Xanthan gum, a microbial biopolymer, is frequently used in microbial enhanced oil recovery field testing (195, 558), often with base-hydrolyzed polyacrylamide as a copolymer. Desirable properties of polymers for microbial enhanced oil recovery include shear stability, high solution viscosity, compatibility with reservoir brine, stable viscosity over a wide range of pH, temperature, and pressure, and resistance to biodegradation in the reservoir environment (195, 286, 539). Organic acids produced through fermentation readily dissolve carbonates and can greatly enhance permeability in limestone reservoirs, and attempts have been made to promote their anaerobic production (589). Organic solvents and dissolved CO<sub>2</sub> can decrease oil viscosity. Fermentation gases can repressurize wells, leading to displacement and production of light or conventional crude oil through a revitalized gas-driven mechanism (589).

Residual oil in reservoirs can be recovered when highly permeable watered-out regions of oil reservoirs are plugged with bacterial cells and biopolymers (584). Bacteria and nutrients are injected into the reservoir, and the system is shut in to allow the biomass to plug the more permeable region as it grows (280, 585). Water is then injected (water flooding) to

force oil trapped in less permeable regions of the reservoir out into the recovery well. A porous glass micromodel has been used to simulate biomass plugging with *Leuconostoc mesenteroides* under nutrient-rich conditions (329, 360, 584, 585, 671). As nutrients flow through the porous glass, a biomass plug establishes at the nutrient-inoculum interface. High substrate loading and high pH promoted plug development (671). The residual oil remaining after water flooding is a potential target for selective reservoir plugging of porous rocks with in situ bacterial growth on injected nutrients (195, 289). Bacteria may exert a much greater plugging effect when they multiply within the reservoir rock rather than when they are injected and accumulate at the surface.

Added or in situ-produced biosurfactants, which aid oil emulsification and detachment of oil films from rocks, have considerable potential in microbial enhanced oil recovery processes (41, 42). Emulsan reduced the viscosity of Boscon heavy crude oil from 200,000 cP to 100 cP, facilitating heavy oil pumping (246). Biosurfactant from the thermo- and halotolerant species, *Bacillus licheniformis* isolates and thermotolerant *Bacillus subtilis* strains have been tested for with various levels of success in reservoirs and in laboratory simulations (285, 385, 400, 674, 675).

In a field microbial enhanced oil recovery study in the Southeast Vassar Vertz Sand Unit salt-containing reservoir in Oklahoma, nutrient injection stimulated growth of the microbial populations, including several aerobic and anaerobic heterotrophic bacteria, sulfate-reducing bacteria, and methanogenic halophiles. Nutrient-stimulated microbial growth produced a 33% drop in the effective permeability in an injection well at North Burbank Unit in Oklahoma, plugging off high-perme-

TABLE 7. Potential microorganisms with petroleum deemulsification properties

Microorganism	Petroleum oil emulsion tested	Emulsion type	Reference(s)
<i>Acinetobacter calcoaceticus</i>	Kerosene-water model; oilfield emulsion	Water-in-oil; oil-in-water	449
<i>Acinetobacter radioresistans</i>	Kerosene-water model	Water-in-oil	449
<i>Aeromonas</i> sp.	Kerosene-water model	Oil-in-water	455
<i>Alteromonas</i> sp.	Kerosene-water model	Oil-in-water	455
<i>Alcaligenes latus</i>	Kerosene-water model	Water-in-oil	449
<i>Corynebacterium petrophilum</i>	Kerosene-water model; crude oil-water	Water-in-oil	161, 583
<i>Bacillus subtilis</i>	Crude oil-water model	Oil-in-water	283
<i>Micrococcus</i> sp.	Kerosene-water	Oil-in-water; water-in-oil	141
<i>Nocardia amarae</i>	Kerosene-water model; oilfield emulsion	Water-in-oil; oil-in-water	95, 346
<i>Pseudomonas carboxydohydrogena</i>	Kerosene-water model	Water-in-oil; oil-in-water	449
<i>Rhodococcus aurantiacus</i>	Kerosene-water model	Water-in-oil; oil-in-water	503
<i>Rhodococcus rhodochrous</i>	Kerosene-water model	Water-in-oil; oil-in-water	667
<i>Rhodococcus rubropertinctus</i>	Kerosene-water model	Water-in-oil; oil-in-water	345
<i>Torilopsis bombicola</i>	Oilfield emulsions	Water-in-oil	161
Mixed bacterial culture	Kerosene-water model; oilfield emulsion	Water-in-oil; oil-in-water	448

ability layers and diverting injection fluid to zones of lower permeability and higher oil saturation (287). In contrast to the poor experience with exogenous organisms for bioremediation (bioaugmentation), injection of selected microbial species into oil field plots in Japan and China resulted in improved oil recoveries of 15 to 23% (248, 680). In one case microbial treatment caused some degradation of long-chain aliphatic hydrocarbon chains but with no apparent degradation of aromatic ring structures.

More than 400 microbial enhanced oil recovery field tests have been conducted in the United States alone, mostly as single-well stimulation treatments on low-productivity wells, so that reliable data are sparse (319, 320, 589). Reservoir heterogeneity significantly affects oil recovery efficiency. Microbial enhanced oil recovery technology may be attractive to independent oil producers, who mostly operate "stripper wells" (producing an average of 0.2 to 0.4 ton of oil per day), of which there are about 470,000 in the United States. A single-well stimulation treatment might increase the rate of production from 0.2 to 0.4 ton of oil per day and sustain the increased rate for 2 to 6 months without additional treatments.

The microbial enhanced oil recovery process may modify the immediate reservoir environment in a number of ways that could also damage the production hardware or the formation itself (280). Certain sulfate reducers can produce  $H_2S$ , which can corrode pipeline and other components of the recovery equipment.

Despite numerous microbial enhanced oil recovery tests, considerable uncertainty remains regarding process performance. Ensuring success requires an ability to manipulate environmental conditions to promote growth and/or product formation by the participating microorganisms. Exerting such control over the microbial system in the subsurface is itself a serious challenge. In addition, conditions vary from reservoir to reservoir, which calls for reservoir-specific customization of the microbial enhanced oil recovery process, and this alone has the potential to undermine microbial process economic viability.

Microbial enhanced oil recovery systems currently represent high-risk processes to oil producers looking for efficient and predictable oil recovery. Modeling approaches which can simulate reservoir conditions and facilitate the development of

more reliable oil recovery strategies may represent a small but uncertain ray of hope, but progress in this area is slow. Development of a universal additive mixture, consisting of a combination of microbial strains, nutrients, surfactants, and buffering agents in appropriate proportions, may represent a further productive line of research.

### Microbial Deemulsification

Oilfield water-in-oil emulsions, formed at various stages of exploration, production, and oil recovery, represent a major problem for the petroleum industry (48, 362, 404, 551). These emulsions are characterized according to their stability as tight (microemulsion, very fine droplets of around 100 Å, hard to break) or loose (coarse droplets, size around 5 µm, unstable, easily broken) (48, 362). Water and dirt in crude oil cause corrosion and scaling on pipelines and reactors, and a maximum sediment and water content of 0.5 to 2.0% is required for pipeline-quality oil (375, 580). To produce saleable oil, petroleum water-in-oil emulsions must be destabilized by costly physical and/or chemical methods.

Microbial species including *Nocardia amarae* (95), *Corynebacterium petrophilum* (583), *Rhodococcus auranticus* (503), *Bacillus subtilis* (283), *Micrococcus* spp. (141), *Torilopsis bombicola* (161), and *Pseudomonas*- and *Acinetobacter*-containing mixed bacterial cultures (448, 449, 653) exhibited deemulsification capabilities (Table 7). Microorganisms generally exploit petroleum hydrocarbon-induced hydrophobic cell surfaces or hydrophobic/hydrophilic properties of biosurfactants to displace or alter the emulsifiers that are present at the oil-water interface (41, 345, 346, 449), although some organisms grown on nonpetroleum hydrocarbon substrates also deemulsified petroleum emulsions (161, 283, 343). Some biologically produced agents such as acetoin (283), polysaccharides, glycolipids, glycoproteins, phospholipids, and rhamnolipids (345) destabilized petroleum emulsions. Surfaces of bacterial cells were responsible for the major deemulsifying activity of *Nocardia amarae* (346) and the mixed bacterial culture (448, 449).

In pure-culture deemulsification studies with pure bacterial cultures, the relationship between initial rate of deemulsification and cell concentration was linear, while that between the extent of deemulsification and cell concentration was logarithmic.

mic (95, 141, 448). A positive correlation was observed between cell concentration and rate of deemulsification by *C. petrophilum* (161) and *Micrococcus* spp. (141).

Emulsion-breaking activity was not affected by lyophilization or freezing/thawing, but was destroyed by autoclaving (448), whereas the deemulsifying properties of *N. amarae*, *R. aurantiacus*, and *R. rubropertinctus* were resistant to autoclaving (344, 345). Alkaline methanolysis destroyed bacterial cell deemulsification ability (345). Washing the cells with any lipid-solubilizing solvent yielded a decrease in their deemulsification capability for water-in-oil emulsions.

The microbial deemulsification rate varies with differences in emulsion composition. Pure cultures of *N. amarae*, *C. petrophilum*, and the yeast *T. bombicola* deemulsified water-in-oil petroleum emulsions diluted with toluene (161, 583). The high viscosity of the emulsion prevented pure bacterial isolates from causing significant deemulsification by *N. amarae* or *R. rhodochrous* (667). Elevating the temperature, which reduces apparent viscosity, generally accelerates deemulsification (344, 430). Microbial deemulsification with a mixed bacterial culture was highest at 50°C (449).

The above discussion raises the question of how some microbial species known to produce biosurfactants and promote petroleum emulsion formation and also some bioemulsifiers, such as rhamnolipids, participate in deemulsification. While the processes involved are undoubtedly complex, microbial deemulsifying activity has generally been observed in water-in-oil emulsions, whereas microbial bioemulsification processes occur during microbial oil biodegradation in oil-in-water emulsions. These are very different physical states, as demonstrated by the fact that chemical surfactants which stabilize oil-in-water emulsions are not effective in stabilizing water-in-oil emulsions and vice versa. Indeed surfactants effective in stabilizing oil-in-water and water-in-oil emulsions have different hydrophilic-lipophilic balances.

Deemulsification of water-in-oil emulsions requires the hydrophilic cell surfaces which exist around cells growing exponentially and in early stationary phase, whereas deemulsification of oil-in-water emulsions requires hydrophobic surfaces produced during the endogenous metabolic phase (345). This suggests that different physiological properties support deemulsification of oil-in-water and water-in-oil emulsions. Additionally, emulsification in a continuous aqueous phase (oil-in-water) is very much a dynamic aerobic microbial growth process. In contrast, in a continuous oil phase (water-in-oil), the low oxygen transfer to microbial cells concentrated in aqueous droplets will limit microbial growth. Any deemulsification effect will likely be due to the predominantly nongrowing cells which were added as an inoculum to the system.

Generally, physicochemical deemulsification processes are capital intensive, and emulsions often generated at the wellhead have to be transported to central processing facilities. Because of the characteristic ability of microorganisms to exert their effects at nonextreme conditions, an effective microbial deemulsifier could be used directly to treat emulsions at the wellhead, thus saving on transport and high capital equipment costs. However, due to the great variability among the properties of crude oil emulsions, inconsistencies are experienced in the performance of all deemulsification processes, physical, chemical, and biological. Further research on microbial

deemulsification processes needs to be aimed at the development of more reliable and universally effective systems.

### Microbial Desulfurization

Sulfur is usually the third most abundant element in crude oil, normally accounting for 0.05 to 5%, but up to 14% in heavier oils (580, 139, 610). Most of the sulfur in crude oil is organically bound, mainly in the form of condensed thiophenes, and refiners use expensive physicochemical methods, including hydrodesulfurization to remove sulfur from crude oil (557). These high costs are driving the search for more efficient desulfurization methods, including biodesulfurization (201, 387, 554). In developing a lower cost biologically based desulfurization alternative, promoting selective metabolism of the sulfur component (attacking the C-S bonds) without simultaneously degrading the nonsulfur (C-C bonds) fuel components in organic sulfur will be the most important consideration (201, 352).

Aerobically grown strains, such as *Rhodococcus erythropolis* and related species, remove the sulfur from compounds such as dibenzothiophene (DBT) without degrading the carbon ring structure (325). These strains can use sulfur from DBT as a sole source of sulfur, which facilitates a strategy for isolation of desulfurizing organisms. Other aerobic selective desulfurizing microbes include *Nocardia* spp., *Agrobacterium* sp. strain MC501 (130), *Mycobacterium* spp. (452), *Gordona* sp. strain CYKS1 (218), *Klebsiella* spp. (157), *Xanthomonas* spp. (131), and the thermophile *Paenibacillus* (341).

*Rhodococcus* sp. strain IGTS8 was isolated from a mixed culture obtained from a sulfur-limited continuous-culture system capable of using organically bound sulfur (293, 434, 469). Strain IGTS8 converts DBT to dibenzothiophene-5-oxide (DBTO), then to dibenzene-5,5-dioxide (DBTO<sub>2</sub>), then to 2-(2-hydroxybiphenyl)-benzenesulfinate (HPBS), and finally to 2-hydroxybiphenyl (HBP) to release inorganic sulfur (464, 465) in a pathway involving two monooxygenases and a desulfinase (224). This enzyme system also transforms alkyl- and aryl-substituted DBT (373). Since the HBP product partitions into the oil phase, its fuel value is not lost. The flammability and explosive risks from the above oxygen-requiring process have led to consideration of cloning the desulfurization genes into anaerobic hosts, which would hyperproduce the enzymes for addition to the crude oil. Desulfurization rates for nonengineered *Rhodococcus* spp. are 1 to 5 mg of HBP per g of dry cells per h, with 55 to 75% of the DBT being released as HBP (309).

Strain IGTS8 exhibits little activity towards thiophenes and benzothiophenes, so new biocatalysts with broad substrate specificity need to be engineered (32). Improved biocatalysts have been engineered, and the desulfurization genes have been manipulated (224, 416, 469, 500, 562). The desulfurization genes of IGTS8 have been characterized, and directed evolution and gene shuffling approaches have broadened their substrate specificity. Strains with deletions of the gene encoding dibenzothiophene sulfone monooxygenase (DszA) or hydroxybiphenyl benzene sulfinate (DszB) in the biodesulfurization pathway (Fig. 4) have been prepared, allowing possible production of potentially valuable sulfur-containing metabolic intermediates as products. Thus, new biocatalysts lacking DszB

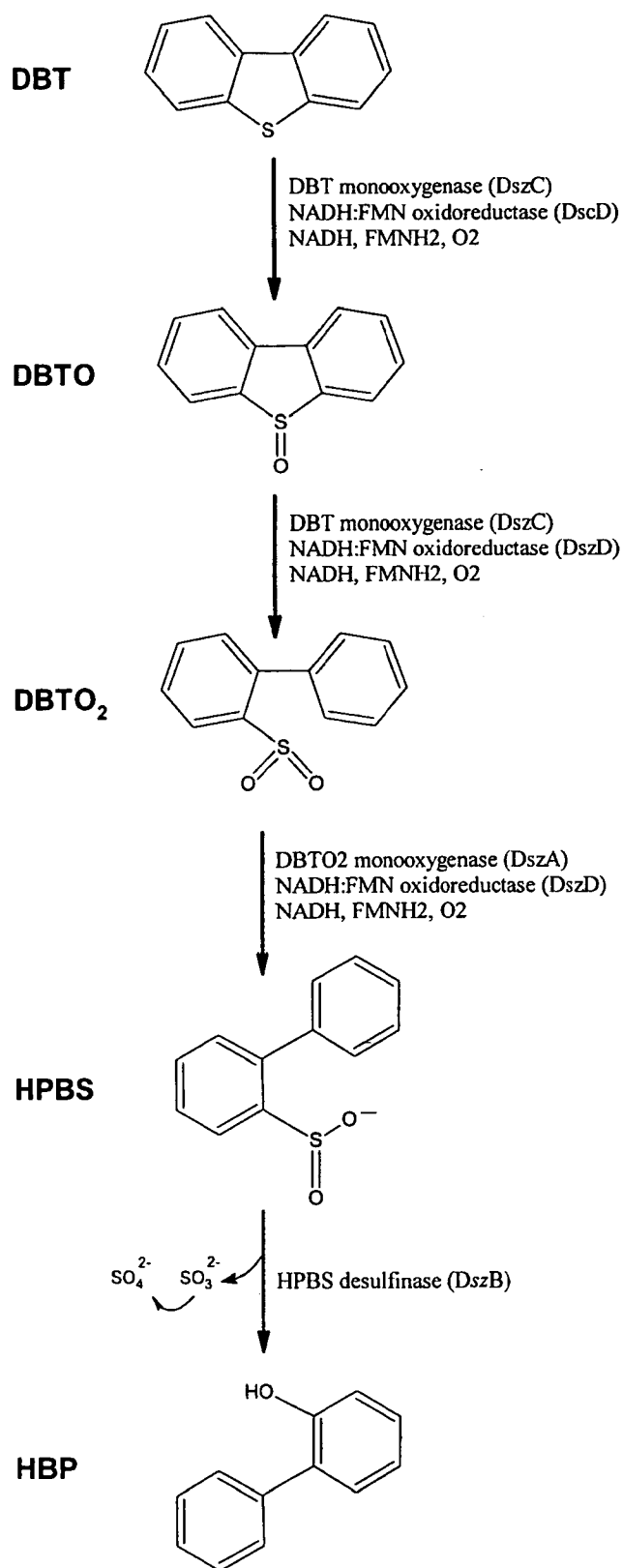


FIG. 4. Proposed sulfur-specific pathway for dibenzothiophene (DBT) desulfurization by *Rhodococcus* species. Abbreviations: DBTO, dibenzothiophene sulfoxide; DBTO<sub>2</sub>, dibenzothiophene sulfone; HPBS, hydrophenyl benzene sulfinate; HBP, hydroxy biphenyl. The *Rhodococcus* pathway does not continue to intermediary metabolism

or DszA stopped desulfurization at the sulfinate or sulfone step for the generation of saleable products with higher desulfurization rates (435). The recombinant *Rhodococcus* sp. strain T09, constructed with a *Rhodococcus-Escherichia coli* shuttle vector, utilized both DBT and benzothiophene as the sole sulfur source (413). The recombinant cells were able to desulfurize alkylated DBT and benzothiophene and also alkylated DBT in an oil-water, two-phase resting cell reaction.

The general water needs of microbial cells require the creation of a two-phase biodesulfurization system with high interfacial areas through energy-intensive mixing and/or addition of a surfactant, with a postdesulfurization deemulsification step. Defining a cost-effective two-phase bioreactor system with subsequent oil-water separation and product recovery represents a key challenge to the viability of biodesulfurization processes (435). Multiple-stage air-lift reactors reduced mixing costs and promoted mass transfer (469), while centrifugal methods were effectively used to break the emulsion, recover the desulfurized oil, and recycle the cells (682).

Since the oxygen-requiring desulfurization enzymes in *Rhodococcus* sp. strain IGTS8 are associated with the external hydrophobic membrane surfaces (148, 311, 459), it has been hypothesized that the enzymes should be active in nonaqueous media. The maximum oil-water ratio for desulfurization was found to be 1.25 ml/g. However 82% sulfur removal was obtained at a 9:1 oil-water ratio (476). Surfactants stimulated biodesulfurization in oil-water systems (476).

Critical aspects of the biodesulfurization process development include reactor design, product or by-product recovery, and oil-water separation. New concepts include the use of multistaged air-lift reactors to reduce the cost of mixing and overcome poor reaction kinetics and to achieve continuous growth and regeneration of the biocatalyst in the same system rather than in a separate reactor (434). Tight emulsions, formed by good oil-cell-water contact and mixing, can be separated continuously with hydrocyclones to obtain relatively clean oil and water. Compared to aqueous systems, biodesulfurizations carried out in two-phase aqueous-alkane solvent systems (309, 460, 461, 469) exhibited increased sulfur removal rates. The extent of biodesulfurization varied with the nature of the oil feedstock, ranging from around 20 to 60% for crude oil and light gas oil (109, 469, 488) to 30 to 70%, 40 to 90%, 65 to 70%, and 75 to 90% for middle distillates, diesel, hydro-treated diesel, and cracked stocks, respectively (469, 488). Hence, the problems of creating two-phase oil-water systems for biodesulfurization of viscous crude oils are circumvented by using more refined products, such as diesel or gasoline (436).

The 1990 Clean Air Act Amendment set the sulfur content of diesel fuel at a maximum of 500 ppm (623), but future values for diesel fuel may be as low as 30 ppm (624). Existing microbial desulfurization technology is not cost effective for heavy or middle distillates of crude oil (416), and hydrodesulfurization technologies cannot achieve the 30-ppm levels required in the

and stops with the release of hydroxy biphenyl, and therefore no decrease in carbon content occurs (435). The physiological significance of the pathway is to obtain sulfur for growth. DszA, DszB, DszC, and DszD are the catalytic gene products of *dszA*, *dszB*, *dszC*, and *dszD*, respectively.



TABLE 8. Microorganisms with potential petroleum-biorefining activities

Biorefining process	Biocatalyst	Microorganism	Reference(s)
Desulfurization	Aerobic bacteria	<i>Rhodococcus erythropolis</i> H2	461
		<i>Arthrobacter</i> sp.	372
		<i>Corynebacterium</i> sp. strain SY1	465
		<i>Nocardia</i> sp.	434
		<i>Agrobacterium</i> sp. strain MC501	130
		<i>Mycobacterium</i> sp. strain G3	452
		<i>Gordona</i> sp. strain CYKS1	218
		<i>Klebsiella</i> sp.	157
		<i>Paenibacillus</i> sp.	341
		<i>Pseudomonas alcaligenes</i>	242
		<i>Rhodococcus</i> sp. strain IGTS8	293
		<i>Rhodococcus</i> sp. strain ECRD-1	229
		<i>Xanthomonas</i> sp.	131
		<i>Desulfovibrio desulfuricans</i> M6	328, 331
	Anaerobic bacteria		
Denitrogenation	Aerobic bacteria	<i>Pseudomonas ayucida</i> IGTN9m	326
		<i>Pseudomonas aeruginosa</i>	6
		<i>Pseudomonas</i> sp. strain CA10	542, 543
		<i>Pseudomonas putida</i> 86	480
		<i>Pseudomonas stutzeri</i>	563
		<i>Rhodococcus</i> sp. strain B1	480
		<i>Comamonas acidovorans</i>	619
		<i>Comamonas testosteroni</i>	546
		<i>Nocardioideis</i> sp.	511
Demetalation	Chloroperoxidase	<i>Caldariomyces fumago</i>	188, 429
	Cytochrome c reductase; heme oxygenase	<i>Bacillus megaterium</i> , <i>Escherichia coli</i>	673

future. However, a combination of biodesulfurization and hydrodesulfurization technology has the potential to achieve these levels (229).

The activities of key enzymes in the desulfurization pathway have also been increased 200-fold (223, 224, 469, 500). A preliminary process design aimed at reducing the sulfur content of gasoline from 1,000 ppm to 100 ppm has been described (622). Gasoline-tolerant bacteria containing the desulfurizing enzyme are available. Any process for gasoline biodesulfurization must achieve costs below the predicted 1.5 cents/gallon cost estimate for alternative innovative chemical-physical desulfurization processes (32).

### Microbial Denitrogenation

Crude oil contains about 0.5 to 2.1% nitrogen, with 70 to 75% consisting of pyrroles, indoles, and carbazole nonbasic compounds. Carbazole is a potent inhibitor of hydrodesulfurization, poisons cracking catalysts, is both toxic and mutagenic, and contributes to the formation of undesirable air-polluting nitric oxides (55, 580). Nitrogenous compounds are generally eliminated from petroleum by expensive hydrotreatment under high temperatures and pressures.

Several species of bacteria that can utilize indole, pyridine, quinoline, and carbazole and its alkyl derivatives have been isolated and characterized, including *Alcaligenes*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Comamonas*, *Mycobacterium*, *Pseudomonas*, *Serratia*, and *Xanthomonas* (6, 66, 190, 222, 299, 311, 457, 480, 511, 563, 619). Bacteria exhibit some general similarities in the pathways for the transformation of aromatic compounds. Oxygenases play an important role in the initial attack in the transformation of nitrogen compounds (187, 299,

545, 546). The initial enzymatic conversion steps yield dihydroxylated intermediates, which then follow either a *meta*- or an *ortho*-pathway, leading to intermediates of central metabolic pathways. Pyrrole and indole are easily degradable, but carbazole is relatively resistant to microbial attack. Recently, selective removal of nitrogen from quinoline by *Pseudomonas ayucida* IGTN9m was reported (326). Increasingly stringent regulations on the nitrogen content of fossil fuels will require very low levels of these heteroaromatic compounds.

Sato et al. (542, 543) identified and cloned the genes responsible for carbazole degradation by *Pseudomonas* sp. strain CA10. To investigate the substrate specificity of the *carA* gene product, a plasmid bearing the *carAa*, *carAc*, and *carAd* genes and expressing only *carA*-encoded proteins was constructed. When introduced into *E. coli*, the recombinant strain was able to transform a wide range of aromatic compounds, including carbazole, *N*-methylcarbazole, *N*-ethylcarbazole, dibenzofuran, dibenzothiophene, dibenzo-*p*-dioxin, fluorene, naphthalene, phenanthrene, anthracene, and fluoranthene.

The major barrier to using a microbial process to remove nitrogen from crude oil is the same as that for desulfurization, namely, the need to create an oil-water two-phase system. Removal of nitrogen and sulfur requires specific attack of the C-N and C-S bonds, respectively, but not C-C bond attack, thus preserving the fuel value of the residual products. To make economic sense, denitrogenation processes need to be integrated with a crude oil desulfurization step (55). However, more recent wisdom has been to retain the hydrodesulfurization technology for initial desulfurization and denitrogenation, with use of microbial desulfurization to further reduce the sulfur level in refined fuels such as diesel and gasoline. Micro-

TABLE 9. Bacterial biosensors for monitoring petroleum contaminants

Bacterial biosensor	Contaminant	Reporter gene fusion	Reference
<i>Pseudomonas fluorescens</i> HK44	Naphthalene	<i>nahG-luxCDABE</i>	252
<i>Pseudomonas putida</i> RB1401	Toluene, xylene	<i>xylR-luxCDABE</i>	89
<i>Pseudomonas putida</i> B2	BTEX	<i>tod-luxCDABE</i>	25
<i>Pseudomonas putida</i> TVA8	BTEX	<i>tod-luxCDABE</i>	25
<i>Escherichia coli</i> DH5 $\alpha$	Alkanes	<i>alkB-luxAB</i>	586
<i>Escherichia coli</i> DH5 $\alpha$	BTEX	<i>xylR-luc</i>	668
<i>Escherichia coli</i>	Benzene derivatives	<i>xylS-luc</i>	273

organisms with potential biorefining activities are shown in Table 8.

### Enzymatic Upgrading of Petroleum Fractions and Pure Hydrocarbons

The unique regio- and stereospecificity properties of enzymes combined with their ability to catalyze reactions in non-aqueous media opens up opportunities to exploit enzyme technology in petroleum processing.

Stereoselective biocatalytic hydroxylation reactions, cytochrome p450-dependent monooxygenases, dioxygenases, lipooxygenases, and peroxidases (114, 259) have tremendous potential for enantiospecific conversions involving petrochemical substrates and their derivatives. Naphthalene dioxygenase (NDO) can produce a range of attractive diol precursors for chemical synthesis and also catalyzes a variety of other oxidations, including monohydroxylation, desaturation, O- and N-dealkylation, and sulfoxidation (339). Because of its broad specificity towards a wide range of aromatic hydrocarbons, NDO can produce chiral petrochemical-based precursors for the synthesis of specialty chemicals (70, 216, 509, 510). Chiral cyclohexadiene diols are potential precursors for the enantio-specific synthesis of many bioactive molecules, and toluene dioxygenase has been used for biosynthesis of enantiomers of erythrose (78, 99, 266, 555, 665). *cis*-Chlorodihydrodiol is an extremely versatile synthon (265). Furthermore, NDO and toluene dioxygenase sometimes form opposite enantiomers of the same product from the same substrate (339).

Epoxides are produced by the action of some monooxygenases, especially the cytochrome P450 monooxygenases, as well as in other epoxidations occurring in biosynthetic pathways (1). Chiral alkane epoxides are synthons for a variety of different syntheses. The alkane hydroxylase and xylene oxygenases of *P. putida* are versatile monooxygenases for stereo- and regioselective oxidation of aliphatic and aromatic hydrocarbons (600, 672). Epoxide hydrolases can transform the resulting epoxides into diols (353). The alkane hydroxylase of *P. oleovorans* has broad specificity and can convert a range of alkanes, alkanols, alkanals, alkenes, and other substrates into interesting products in two-phase systems (548, 628). For some systems, bioconversion rates producing chemical products in the cost range of US\$3 to US\$10 per kg have been predicted (670).

A recombinant *E. coli* strain containing the *P. oleovorans alk* genes was able to grow on sugars in the presence of a bulk *n*-alkane phase and convert octane to the corresponding octanoic acid (184). To overcome degradation of the products of the xylene monooxygenase from *P. putida*, the *xyl* genes from the TOL plasmid encoding this enzyme may be inserted in *E.*

*coli* (672). *P. oleovorans* can convert octane to medium-chain poly(3-hydroxyalkanoates), with potential for use in biodegradable plastics (247), at projected large-scale manufacturing costs of less than US\$10 per kg (348).

A number of oxidative enzymes have been the target of directed evolution (115, 116). Cytochrome P450<sub>cam</sub> monooxygenase from *P. putida* has successfully evolved to function more efficiently in the hydroxylation of naphthalene (516), and dioxygenases with improved thermostability and substrate specificity have been designed (207, 324, 354, 463).

In active hybrids of naphthalene and 2,4-dinitrotoluene dioxygenase enzyme systems, replacement of small subunits affected the rate of product formation but had no effect on the substrate range, regiospecificity, or enantiomeric purity of oxidation products with the substrates tested (474). Substitution of valine or leucine for Phe-352 near the active site iron in the  $\alpha$ -subunit of NDO altered the stereochemistry of naphthalene *cis*-dihydrodiol formed from naphthalene and also changed the region of oxidation of biphenyl and phenanthrene (473, 475).

New protein engineering developments will undoubtedly result in the creation of powerful biocatalysts with applications for specific transformations or upgrading of petroleum fractions or pure hydrocarbon compounds. Such developments have already occurred with simpler biocatalytic systems, such as the extracellular microbial enzymes.

However, in general, the use of enzymes in synthesis has to exploit the main competitive advantage of enzyme over chemical methods, namely, for stereo- and regiospecific synthesis, producing single isomeric products. This limits the range of reactions, usually to production of bioactive compounds or precursors, while biocatalytic systems in non-aqueous-phase media has extended the range of substrates accessible to enzymes to include hydrophobic petroleum compounds; reaction rates in non-aqueous-phase media are often much lower than in aqueous systems. These drawbacks limit the applicability of this technology to specialty chemicals and steer it away from bulk petroleum processing.

### BACTERIAL BIOSENSORS

Bacterial biosensors uniquely measure the interaction of specific compounds through highly sensitive biorecognition processes and offer great sensitivity and selectivity for the detection and quantification of target compounds (315, 608). Whole-cell biosensors, constructed by fusing a reporter gene to a promoter element induced by the target compound, offer the ability to characterize, identify, quantify, and determine the biodegradability of specific contaminants present in a complex mixture without pretreatment of the environmental samples

(11, 142, 179, 483). The genetic information, located on a plasmid vector, is inserted into a bacterial strain so that the engineered fusion replicates along with the cell's normal DNA. Biosensor systems include a wide range of integrated devices that employ enzymes, antibodies, tissues, or living microbes as the biological recognition element. Bacterial biosensors developed for monitoring petroleum contaminants are shown in Table 9.

There is a continuing need to monitor the concentration, transformation, and toxicity of common soil and groundwater pollutants, including petroleum contaminants such as BTEX and PAH compounds in the environment. Many current analytical techniques used for monitoring pollutants require expensive equipment and extensive pretreatment of the environmental samples. The inherent difficulties in classical analytical methods have created an interest in the development of alternative methods, including novel bacterial biosensors. These biosensors offer significant advantages over conventional analytical methods. Classical analytical methods cannot distinguish between unavailable and bioavailable compounds. While conventional analytical methods provide information about concentrations in the contaminated phases, they do not assess the bioavailability of a contaminant, which is an important consideration of site remediation (11). Bacterial biosensor measurements have also been shown to be within very close range of those measured by standard gas chromatography-mass spectroscopy techniques (e.g., 3% in the case of toluene) (668).

The presence of toxic compounds and the potential associated ecological risks can be determined by using bacterial biosensor and toxicity tests. Although several biochemical and genetic methods which give clear signal or bands are available, data on field environmental quality assessment are limited. There are some outstanding questions. Are microbes capable of degrading the particular pollutant present in the contaminated site and will the biological treatment method effectively remove the contaminants? What happens if the concentration of the contaminant is low compared to that of other biodegradable or metabolizable substrates? Although these questions may not be answered, molecular and biochemical tools available today would help provide some of the answers in the coming years.

Broad-specificity biosensors are used for toxicity testing and respond to a wide range of compounds, including petroleum hydrocarbons in contaminated soils, a good example of which is the commercially available Microtox assay, used for measuring the toxicity of environmental samples by monitoring the light production of the naturally bioluminescent marine bacterium *Photobacterium phosphoreum* (89). Since bacterial bioluminescence is tied directly to cellular respiration, any inhibition of cellular metabolism due to toxicity results in a decrease in the light emission of the affected cells. In nonspecific bacterial biosensors, *lux* genes are fused to heat shock promoters so that exposure of the cells to toxic organic compounds or metals rapidly induces light production (142).

With *P. fluorescens* HK44, a prototype bioluminescent catalytic reporter strain, a bioassay for the quantitative assessment of naphthalene and salicylate biodegradation in aqueous, soil, and slurry systems is available (252, 253). A linear relationship was established between substrate concentration and biolumi-

nescence over a concentration range of up to two orders of magnitude, and naphthalene induced a significant response at a concentration as low as 45 ppb. The potential use of immobilized *P. fluorescens* HK44 cells for on-line monitoring of PAH degradation in the subsurface has also been demonstrated (657).

A biosensor for detecting the toxicity of PAHs in contaminated soils was constructed with an immobilized recombinant bioluminescent bacterium, GC2 (*lac::luxCDABE*), which constitutively produces bioluminescence (231). The monitoring of phenanthrene toxicity was achieved through measurement of the decrease in bioluminescence when a sample extracted with the rhamnolipid biosurfactant was injected into a minibioreactor. This system was proposed to be used as an in situ system to detect the toxicity of hydrophobic contaminants in soils and for the performance evaluation of PAH degradation in soils. Several biosensors have been developed for the detection of benzene, toluene, ethylbenzene and xylene isomers (89, 273, 363, 668). *E. coli* HB101 cells harboring engineered plasmid pTSN316 (carrying a transcriptional fusion between firefly *lux* genes and the promoter of the *xylS* gene) were immobilized on the tip of a fiber-optic system with a dialysis polycarbonate membrane were able to detect BTEX compounds and related monoaromatics (ethyltoluene and chlorotoluene) in the ppm range (273). The toluene detection range of *E. coli* cells carrying pGLTUR plasmid (fusion of firefly *lux* genes to transcriptional activator *xylR* gene) was between 10 and 20  $\mu$ M (668). The calculated toluene concentrations were within 3% of those measured by gas chromatography-mass spectroscopy techniques.

To monitor toluene and trichloroethylene cometabolism and kinetics of degradation an on-line monitoring system was developed with *P. putida* B2, which harbors a plasmid with *tod-luxCDANE* transcriptional fusion (26, 316). A linear relationship between bioluminescence and toluene concentrations between 0 and 10 mg/liter was observed in assays of *P. putida* B2 growing cells. The cells immobilized in alginate beads were also able to provide on-line monitoring of biotransformation and cometabolism of toluene and trichloroethylene.

Simpson et al. (570) developed an advanced system consisting of biosensor cells interfaced with an integrated circuit called the bioluminescent bioreporter integrated circuit, which can detect the optical signal, distinguish it from the noise, perform signal processing, communicate the results, and also carry out position sensing. A prototype has been constructed with *P. putida* TVA8 cells with a sensing capacity for toluene vapors at 1 ppm.

A bacterial biosensor for measuring the bioavailable middle-chain-length alkanes was developed (586). *E. coli* DH5 $\alpha$  containing the regulatory gene *alkS* and a transcriptional fusion between the *alkB* promoter and *luxAB* genes on two different compatible plasmids was used. The biosensor responded to octane at concentrations as low as 24.5 nM, with a linear response up to 790 nM. The biosensor cells were capable of sensing a range of other compounds that were structurally related, including linear alkanes from pentane to decane and the branched alkane 3-methylheptane.

Even with the rapid advances in nanotechnology, there are still limitations with the bioluminescent bacterial biosensors. Living cells are complex systems, and light output of the bi-

oluminescent biosensors depends not only on the chemical complexity of the sample but also on variations of the physiological state of the cells, including changes in the rate of gene transcription, protein synthesis, membrane permeability, and metabolism. Over the last decade, advances have been made in the use of molecular diagnostics in bioremediation. Qualitative detection methods have been replaced with methods that provide quantitative measurements of specific microbial populations present in the contaminated sites. To assess the microbial treatment of petroleum-contaminated sites, the bioavailable concentration of pollutants could be measured with bacterial sensors and the overall genetic potential of the degradative pathways determined by DNA tests. It could also be verified whether the pollutant concentrations are sufficiently high to induce the particular degradation. However, the validity of these methods needs to be tested in the field to assess the practicability and usefulness of these techniques in bioremediation. The commercialization of biosensors for environmental applications has shown only modest progress over the last 5 years. The advances in nanotechnology will continue to result in higher sensitivity and more versatile operational characteristics. Nevertheless, whole-cell biosensors hold a great deal of promise for continuous online monitoring of pollutants in environmental applications.

## CONCLUSIONS AND FUTURE PROSPECTS

Our review of hydrocarbon metabolism illustrates how molecular tools are contributing to substantially advance our knowledge of the intricate mechanisms of transformation of hydrocarbons. Because of the more challenging methodologies involved in implementing research on anaerobic microbial hydrocarbon degradation, our understanding of this area has lagged behind that of aerobic systems, and great opportunities exist to further elucidate anaerobic hydrocarbon cellular processing mechanisms. These metabolic studies, both aerobic and anaerobic, will in turn provide a greater insight into novel biocatalytic mechanisms.

At least in the context of a perspective that microbes generally thrive in aqueous environments, the hydrophobic nature of hydrocarbons represents a physiological challenge to microbial systems to address hydrocarbon accession. Detailed mechanisms of hydrocarbon uptake and efflux have only recently been reported. Excellent advances in our knowledge of active hydrocarbon efflux, mediated by different efflux pumps, have recently been made. While evidence exists that some of the processes of hydrocarbon uptake are energy dependent, molecular mechanisms for active hydrocarbon uptake have not been established. Further studies in these areas will undoubtedly lead to exciting new findings and add an important dimension to the overall scientific quest to better understand all cellular transport mechanisms.

The biochemical basis of bacterial chemotaxis has been studied for water-soluble systems. However, little is known about taxis as it applies to the mechanisms used by hydrocarbon-degrading bacteria addressing water-insoluble substrates. Nevertheless, preliminary evidence for chemotaxis has been provided, suggesting that exciting opportunities exist to probe the underlying mechanisms involved.

Studies of community dynamics related to petroleum-de-

grading microbes have the potential, *inter alia*, to enhance our understanding of the roles played by microbes in the natural genesis of petroleum over geological time and on the long-term effects of petroleum pollution and to determine new remediation and waste treatment approaches. These studies provide insights into the awesome diversity of microbial populations, and accelerated molecular and genomic methodologies and more automated techniques will undoubtedly lead to the characterization of exciting new microbial strains and biocatalytic activities. Apart from adding to our understanding of the complexities of these natural communities, the strains and their metabolic capabilities will surely find new applications in microbial technology.

The bioremediation component of this review focused on treatment of high-volume hydrocarbon wastes. The data show that conventional landfarming of these wastes leaves substantial proportions of the constituent hydrocarbons, including the highly toxic high-molecular-weight PAHs, undegraded. Evidence is also provided that in landfarming practices, as in many conventional bioremediation systems, a large fraction of the volatile hydrocarbons is not biodegraded but is rather transferred to the atmosphere through volatilization. An increasing focus on regulation and control of volatile organic carbon emissions calls for hydrocarbon remediation and waste treatment systems which contain or destroy the volatile organic carbon fraction. These environmental requirements provide scope to microbiologists to establish bioreactor-based environments in which oily soil slurries and sludges may be treated, with volatile organic carbon containment, and where rates and extents of hydrocarbon degradation are maximized. Surfactants can be used to support hydrocarbon accession, and there is evidence that retention of the volatile organic carbons as microbial substrates, rather than their volatilization, facilitates biodegradation of some of the more recalcitrant molecules through cometabolism. This review demonstrates that these more optimized systems greatly accelerate biodegradation processes from the rates observed in landfarms (0.5 to 1% of total petroleum hydrocarbon contents per month) to around 1% per day in large-scale bioreactors while achieving endpoint non-hazardous criteria.

Our knowledge of the potential roles of chemical and biosurfactants in accelerating hydrocarbon accession is still very limited. While bioreactor use facilitates volatile organic carbon containment and process optimization and control, this remediation approach would also enable genetically engineered organisms to be exploited for specific bioremediation applications, given that we are still left with discretion over their fate in the bioreactor-treated material.

Microbial enhanced oil recovery processes mobilize oil in reservoirs through repressurization and viscosity reduction mechanisms. As with *in situ* bioremediation systems, the environment, over which the microbiologist has little control, influences optimal performance. Clearly, microbial products reducing oil viscosity could be produced above ground under optimal conditions and injected with high chances of efficacy, and research on finding microbial products with universal applications in this area is worth pursuing. A more robust universal microbial system for assisting in the repressurizing of porous reservoirs is desirable and should be aided by ongoing modeling studies directed to manipulating simulated porous

reservoirs in columns. These approaches will facilitate implementation of microbe-based research to determine the most desirable strain types, nutritional, metabolic, and physiological characteristics needed to achieve high success rates in application of the microbial technology in oil recovery. However, the future efficacy of improved microbial enhanced oil recovery technology remains very uncertain.

While hydrocarbon-metabolizing bacteria demonstrate efficient emulsification characteristics (oil-in-water emulsions) during oil biodegradation, microbial deemulsification phenomena are typically observed in oil-dominant water-in-oil systems. Biological deemulsification could provide a low-capital-cost solution for treatment of wellhead emulsions. Current studies indicate substantial performance variation with different emulsion compositions and different oils. Research aimed at understanding the nature of these processes and the critical factors influencing deemulsification is needed.

Microbial processes for desulfurization and denitrogenation of crude oil are limited by the challenges of having to operate in a two-phase aqueous-oil system, and desulfurization efforts have shifted towards reduction of the sulfur content of diesel fuel to achieve the lower prescribed sulfur levels in this product. Recent research has resulted in a 200-fold increase in expression of key desulfurization genes in the best strains. The main challenge now relates to broadening of the specificity of the key biocatalysts, which currently exhibit limited activity towards important thiophene and benzothiophene sulfur components.

There is substantial interest in the use of biocatalysis for the synthesis of chiral bioactive compounds and the use of non-conventional aqueous-organic reaction media to accommodate hydrophobic reactants. Recently, the potential for hydrocarbon-transforming oxygenases and other enzymes in enantiospecific biotransformations has been demonstrated. Future research exploiting molecular techniques promises to create a range of novel biocatalysts which could underpin microbial technology for commercial production of high-value enantiomers from petroleum precursors.

On a different front, bacteria with selected petroleum-metabolizing enzymes combined with systems amenable to electronic monitoring, for example, bioluminescence genes, may be used as biosensors to monitor particular petroleum-based environmental pollutants or toxicity in process control applications. This research topic is at an early stage of investigation but represents an important interdisciplinary pursuit encompassing biological and electronic expertise.

Clearly, petroleum microbiology research is advancing on many fronts, spurred on most recently by new knowledge of cellular structure and function gained through molecular and protein engineering techniques, combined with more conventional microbial methods. Improved systems for biodegradation of petroleum components are being commercialized with positive economic and environmental advantages. Ground-breaking work is being done to engineer new biocatalysts for applications in desulfurization and organic synthesis.

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MICROBIOLOGICAL METHOD FOR PRODUCTION OF OIL HYDROCARBONS AND SEPARATE  
HYDROCARBON FRACTIONS FROM SOLID FOSSIL FUELS  
[MIKROBIOLOGICHESKIY SPOSOB POLUCHENIYA UGLEVODORODOV NEFTI I  
OTDEL'NYKH UGLEVODORODNYKH FRAKTSIY IZ TVERDYKH GORYUCHIKH  
ISKOPAYEMYKH]

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The invention concerns methods of processing solid combustible fossils by means of microorganisms and solves the task of producing hydrocarbons identical to those contained in petroleum of natural origin and individual fractions thereof.

The capacity of microorganisms for intracellular synthesis of hydrocarbons (E.G. Dedyukhina, V.K. Yeroshin. Biosintez uglevodorodov mikroorganizmami (Biosynthesis of hydrocarbons by microorganisms). Uspekhi sovremennoy biologii AN SSSR, 1973, vol. 76, no. 3(6), p. 351-361).

A method of microbiological synthesis of oil out of materials of animal origin by treating them at high temperature and pressure ( $P=3-30$  atmospheres;  $t=100-350^{\circ}\text{C}$ ) in the presence of plant residues (DE2455368, application published 1976).

This method is complicated and unsafe, since it requires the use of super-high temperatures and pressure. A hydrocarbon mixture close in composition to petroleum is obtained.

The impossibility of directly controlling the process of producing hydrocarbons is a disadvantage of the method.

A microbiological method of acting on solid fossil fuels with cultures of the bacteria *Thiobacillus aquaesulis*, or *Thiobacillus denitrificans*, or *Thiosphaera pantropha* in any combinations is proposed for producing a wide range of petroleum hydrocarbons of petroleum and individual fractions thereof.

The method consists in treating suspensions of ground raw material (solid fossil fuels) with cultures of *Thiobacillus aquaesulis*, or *Thiobacillus denitrificans*, or *Thiosphaera pantropha*, taken in any combinations, in the presence of compounds that are

hydrogen donors.

The process is conducted at temperatures of 4-60°C (temperature optimum 18-24°C) and at a pH of 6-9.

The following are used as hydrogen donor substances: acids, phenols, naphthalene, tetrahydronaphthalene, cresol.

Solid fossil fuels - humites, anthracite and bituminous coal, peat - are used as the raw material for producing petroleum hydrocarbons.

Bacteria of the species *Thiobacillus thioparus* also are used additionally in the case of using peat as a raw material.

The method is performed in the following way.

Solid fossil fuels are subjected to mechanical crushing, then liquid - water, inorganic (sulfuric, nitric, etc.) acid, or any liquid hydrocarbon - petroleum or any petroleum fraction, is added in order to create a suspension or paste. Weak or more concentrated acids are used. The acids are used as diluting agents (as the liquid phase) and as hydrogen proton donors. Before mixing them with solid fuel in the liquid phase of the acid, a culture of *Thiobacillus aquaesulis* bacteria is added to liquid hydrogen or in petroleum individually or in combinations with bacteria of the species *Thiosphaera pantophora*. Then the liquid and solid phases are mixed once by means of using a mixer, an air pump (barbotation), or gas pump - decanting the suspension, or in another way. The bacterial cultures used are preadapted to hydrocarbon and organic (coal-peat) media.

According to a second version of the method, a bacterial culture is added to a prepared aqueous-organic (water-coal, water-peat, etc.) medium (suspension), to an acid-organic, or to a hydrocarbon-coal

(peat etc.) medium. Previously cultured cultures of bacteria in water, an aqueous solution of acid, or hydrocarbon (petroleum, etc.) according to the first version of the method is technically easier and improves the mass-exchange of the process and therefore is preferably.

The solid fossil fuels are mixed with liquid in order to provide contact of the bacterial culture dissolved in the liquid phase with maximum amount of surface of the solid substrate of the raw material (coal etc.) and in order to provide maximum contact of the bacteria with the macromolecules and molecules of solid fossil fuels.

Organic or inorganic acid in an amount of 1-2% of concentrated 96% acid is added to solid fossil fuel organic material as a hydrogen donor in the system, for example, an aqueous 1-2% solution of acid is added to the solid fossil fuel material in a ratio of 1:1-1.5 (0.75-1 liter of solution per 1 kg of solid fuel). In the case of using water or weak acid solutions as the liquid medium, on the whole the water-organic (coal, peat, etc.) suspension is converted into a water-hydrocarbon emulsion. This emulsion contains water (a mixture of industrial water - diluting agent and water in the form of moisture initially included in the composition of certain high-moisture solid fossil fuels), molecules of individual hydrocarbons, and extensive petroleum fractions, obtained from solid fuel raw material under the action of the above-mentioned bacteria, and also a slurry consisting of coal ash (metals, sulfur, oxidized compounds). Then hydrocarbons (synthetic petroleum) are separated from the water by any traditional industrial methods - a settling period, use of deemulsifiers, separation, etc. It is preferable to use petroleum or any liquid hydrocarbon substances that are added to the solid fossil raw material

in different economic proportions (from 30 to 100% petroleum to the solid fossil fuel material), sufficient for creating a paste (suspension), as the liquid medium for bacteria in order to eliminate the process of dehydration of the synthetic petroleum obtained.

As a result, a homogeneous hydrocarbon material - synthetic petroleum (the set of fractions and individual hydrocarbon substances), the components of which are hydrocarbons and petroleum fractions, obtained under the action of bacteria from solid fuel raw material, and petroleum or a petroleum product, uses as a diluting agent (liquid phase) are obtained as a result. Hydrocarbons obtained from coals are organically mixed with the initially poured petroleum (petroleum product), mutually dissolving, in this case the total volume of the petroleum (petroleum product) is increased correspondingly. The petroleum or petroleum product used at the beginning of the process as a diluting agent (liquid phase), either is removed without mass losses from the synthetic petroleum obtained, or is used together with the latter as a single petroleum raw material (for distillation, chemical processing, heating, and other purposes).

The volumetric ratio of gasoline and diesel fractions included in the petroleum - diluting agent remains unchanged in the course of the entire process.

The amount of benzene, its uninucleate homologues, and isoparaffin compounds is increased in relation to the normal paraffin and naphthalene hydrocarbons in the gasoline fraction of petroleum or any other petroleum product, among them in the pure gasoline fraction, used as a diluting agent (liquid phase), as a result of performing the method, under the action of bacteria of the species *Thiobacillus*



*aquaesulis*. Increasing the amount of the lower (uninucleate) aromatic compound and isoparaffin hydrocarbons in the gasoline fraction leads to an increase in its octane number and fuel grade. The benzene homologues and isoparaffin substances formed are a more valuable raw material than the original compounds: condensed cycles and normal paraffin compounds.

The process time for forming synthetic petroleum and petroleum products depends on the degree of pulverization of the solid fossil fuels, the seeded bacteria concentration, the material exchange indicators (which depend both on the initial and on the subsequent mixing), and the presence of a hydrogen source in the system (hydrogen donor). Optimal parameters are: size of the pieces of solid fossil fuel no more than several cubic millimeters, seeded bacteria concentration not less than  $10^4$  per ml of medium, or 1 kg of dry preparation (several billion bacteria in 1 ml), good initial and subsequent constant mixing (or pure periodic mixing), hydrogen donor concentration in mass volume not less than 1-2% (optimum 4-7%) in the organic mass of the solid fossil fuel (for example, 4-7% solutions of organic or inorganic acids in a volume 1:1 to the mass of coal, or phenol, cresol, naphthalene, or tetrahydronaphthalene in an amount of no less than 1-2 kg per 100 kg of organic material of solid fossil fuel). In the case of the given optimal ("ideal") conditions the process of forming petroleum hydrocarbons from solid fossil fuels takes place in the reaction zone in 6-24 hours. The attainable yield is 90% of the weight of raw material.

The active mechanism of the bacteria is to break down the macromolecules (molecules) of solid fossil fuels which, according to

the traditional data, are very condensed polycyclic and polyaromatic systems. The bacteria break down the carbon-carbon (C-C) bonds and the heteroatomic bonds of solid fossil molecules. As a result less condensed systems of lower molecular weight takes place up to different uninucleate aromatic compounds, petroleum and liquid paraffin hydrocarbons, forming different petroleum fractions. Removing sulfur, nitrogen, and oxygen atoms from the condensed systems, the bacteria break heteroatomic bonds, which leads to breakdown of the condensed systems and reduction of the molecular weight of the latter.

By breaking the oxygen, sulfur, and carbon-carbon bonds and transferring the electrons in the organic material, the bacterial strains used contribute to the formation of free radicals, according to which hydrogen from an external source-hydrogen donor is added (stabilization of radicals with hydrogen). Hydrogenation of solid fossil fuels takes place. In a system with the given species of bacteria the hydrogen donor "transfers" to the solid fossil fuel 5-10% (by weight) of the hydrogen (hydrogen content of the organic material increases by 5-10%). As a result the organic material of the solid fuels is converted into hydrocarbons, in particular into liquid hydrocarbons.

The process of forming low-boiling hydrocarbons, synthetic petroleum (liquid hydrocarbon raw material) and its derivatives from solid fossil fuels, in particular light fractions is performed in three versions, which may be observed and fixed on the super-molecular and visual levels:

1. direct formation of liquid petroleum hydrocarbons of petroleum

(on the chemical molecular level it is possible via the stage of intermediate products) in the form of low-boiling fractions, or in the form of a wide range of fractions - synthetic petroleum, with a yield of 50-90%, calculated according to the dry weight of the coal (the process is performed with peat or brown coal in anaerobic conditions, in aerobic conditions with anthracite coal), in both cases in the presence of a hydrogen donor the process time is reduced two-fold.

2. formation of fuel oil as an intermediate product (process time 2-4 weeks in anaerobic or aerobic conditions) which then in the course of performing the method is subjected to bacterial decomposition of the hydrocarbon molecules (biocracking) and bacterial hydration (biocatalysis) with the formation of more liquid and low-boiling hydrocarbons and petroleum fractions (2-4 more weeks in anaerobic conditions with donor hydrogen);

3. formation of fuel oil, as an end product, with up to a 80-95% yield of the latter as compared with the initial solid raw material - dry weight of coal or other solid fossil fuels (process time 2-4 weeks in anaerobic conditions, or in aerobic conditions, with donor hydrogen or without it).

4. formation of tar (anthracite pitch) analogs with a 90-95% yield as compared with the initial raw material. This intermediate material contains both uninucleate and multinucleate aromatic and petroleum cycles, both liquid and solid paraffin hydrocarbons, phenols, pitches, and neutral substances. The tar obtained according to the given method visually represents a fairly uniform viscous liquid material, as opposed to the precursor already pertaining to the hydrocarbon raw material. The tar can be final products (because of

the artificial stopping of the process), or is further subjected to bacterial decomposition and hydration, with the formation of individual hydrocarbons, included in gasoline, diesel, and fuel oil fractions (in a wide fraction of synthetic petroleum).

In the case where the process is stopped in the tar stage (for example, by limiting the process time - less than 1-2 weeks), the product obtained can serve as fuel or raw material for traditional processes of hydration - destructive hydrogenation (obtaining liquid hydrocarbon fuels and individual substances). It is desirable for liquefaction of the tar to take place by prolonging the process with the addition of a hydrogen donor (in the case of an initial absence thereof). Further destruction of large molecules, hydration and lightening of the molecular hydrocarbon composition with the formation of petroleum fractions takes place. This bacterial process removes heteroatoms - sulfur, nitrogen, oxygen, from the tar molecules, decomposition of pitches and high molecular weight compounds, and conversion of tar into liquid petroleum fractions.

Experiment. Bacteria of the species *Thiobacillus aquaesulis* and *Thiosphaera pantotropha* were adapted to hydrocarbon and coal-peat media, as a result the strains *Thiobacillus aquaesulis*-39 and *Thiosphaera pantotropha*-66, capable of existing in the given media, and utilizing molecules of solid fossil fuels, bitumens and petroleum in their vital activity, were obtained.

In coal-petroleum and water-coal suspensions a culture of *Thiobacillus aquaesulis* bacterial was added separately or in combination with a culture of bacteria of the species *Thiosphaera pantotropha* in the form: pure separated culture; together with a

liquid nutrient medium. The bacterial concentration was taken as being no lower than  $10^4$  bacteria per 1 ml of medium, or 1 kg of separated biomass per 10-60 tons of bituminous or anthracite coal, peat, or any other solid fuel substrates.

A gradual transformation of the molecules of solid fossil fuels (coal, peat, etc.) into hydrocarbon molecules and the formation of hydrocarbons containing synthetic petroleum takes place in the course of 1-2 months without mixing the system.

In the case of an initial introduction of a hydrogen donor substance into the system in an amount of 1-5% of the weight of solid fossil fuel and with constant or partial periodic mixing (5 minutes every 2-4 hours), conversion of solid fossil fuels into hydrocarbons began after 7 hours, the formation of the hydrocarbon material completely stopped after 7 days.

#### Examples

The following forms of raw material were taken for the experiment:

peat of small local deposits in Tataria and peat from commercial depositions in Byelorussia, Ryazan, Tyumen, and Novgorod provinces.

Test sample of peat. Elementary composition, % of original peat material (transitional type of peat, that is, between high and low): C 58.6%, H 6%, O 32.8%, S 0.4%, N 2.2%. True density 1.5. Combustion heat 7.5 MJ/kg.

Bituminous coal from the Moscow coal basin (B2 industrial group), density  $1.5 \text{ g/cm}^3$ , combustion heat 10.9 MJ/kg. Elementary composition of test sample, % of coal fuel weight: C 75%, H 5.5 %, O 18.5%, N 1.9%.

Bituminous coal from the Kansk-Achinsk coal basin (B1 and B2), density 1.6 g/cm<sup>3</sup>, combustion heat 11.8-15.6 MJ/kg. Elementary composition of test sample, % of coal fuel weight: C 75%, H 5.5 %, O 18.5%, N 1.0%. Combustion heat of test sample 6600 kcal/kg.

Bituminous coal from Orenburg province, B1 and B2 (Tyul'ganskiy coal section), density 1.8 g/cm<sup>3</sup>, moisture up to 54%, ash content 24-30%, combustion heat 1850-1900 kcal/kg.

Anthracite coal from the Kuznetsk coal basin, grade D (long flame), G (gas), Zh (liquid), KZh (coke-fatty), K (coke), T (lean).

Elementary composition, % of coal fuel weight:

grade D: C 80%, H 5.5%; O+S org. = 12.7%, N 1.8%. Combustion heat 7950 kcal/kg.

Grade G: C 84%, H 5.0%, O + S 9.3%, N 1.7%. Combustion heat 8200 kcal/kg.

Grade Zh: C 87%, H 5.0%, O+S 6.3%, N 1.5%. Combustion heat 8450 kcal/kg.

Grade KZh: C 87.5%, H 4.9%, O+S 6.1%, N 1.5%. Combustion heat 8500 kcal/kg.

Grade K: C 88%, H 4.8%, O +S 6.0%, N 1.5%. Combustion heat 8600 kcal/kg.

Grade T: C 91%, H 4.0%, O+S 4.0%, N 1.0%. Combustion heat 8500 kcal/kg.

Anthracite coal from the Donetsk coal basis, combustion heat 21.2-26.1 MJ/kg:

Grade D-T and A (anthracite) grade D (long-flame), G (gaseous), Zh (fatty), K (coke), OS (lean caking), T (lean), and A (anthracite).

Elementary composition, % of coal fuel weight:

Grade D: C 80%, H 5.5%, O+S org. 12.7%, N 1.8%. Combustion heat 7950 kcal/kg.

Grade G: C 85%, H 5.0 %, O+S 9.3%, N 1.7%. Combustion heat: 8200 kcal/kg.

Grade Zh: C 87%, H 5.0%, O+S 6.3%, N 1.5%. Combustion heat 8450 kcal/kg.

Grade K: C 88%, H 4.8%, O+S 6.0%, N 1.5%. Combustion heat 8600 kcal/kg;

Grade OS: C 89%, H 4.5%, O+S 5.0%, N 1.5%. Combustion heat 8600 kcal/kg.

Grade T: C 91%, H 4.0%, O+S 4.0%, N 1.0%. Combustion heat 8500 kcal/kg.

Grade A: C 93%, H 3.0%, O+S 3.0%, N 1.0%. Combustion heat 8200 kcal/kg.

The wide range of hydrocarbons obtained (synthetic petroleum) and fuel oil independent of the type of solid fossil fuel practically did not differ from one another according to physical-chemical characteristics, but depending on the type of raw material differed from one another according to the group hydrocarbon composition (that is, according to the aromatic, methane, and petroleum hydrocarbon content). Yield: 1 t of the organic part of solid fossil fuels - 90% hydrocarbons, 1 t of coal + 1 t of 2% acetic acid + 100 g of biomass, yield 800 kg of a wide range of hydrocarbons, 1 t of peat + 1 t of 2% acetic acid + 100 g of bacterial biomass, yield 200 kg of a wide hydrocarbon fraction.

The physical-chemical characteristics of the synthetic petroleum obtained, that is, the entire set of fractions obtained and individual

hydrocarbons (initial material - peat or bituminous coal, or anthracite coal, or fuel shale).

Chromatography of the gasoline fraction

Hydrocarbon content 84%, hydrogen content 14%, sulfur content 1.5%, nitrogen and oxygen content 0.5%.

Density - 0.85-0.95

Viscosity - 5-15 cSt (50°C)

sulfur not more than 1.8% - (on the average 1-1.5%)

resins - 8-15%

asphaltenes - 0.5-1.5%

cokeability - 3-8%

combustion heat - 10400-11000 kcal/kg

Yield of fractions, weight % n.k. 28-200°C=22%, 28-300°C=38%, 28-350°C=50%, above 350°C=50%.

Otherwise (the same): 28-200°C=22%, 200-300°C=16%, 300-350°C=12%, above 350°C=50%.

Differences in the group hydrocarbon composition:

The synthetic petroleum obtained from peats: is characterized by the following group hydrocarbon composition (total content of substances of different classes in all fractions):

Aromatic hydrocarbons 45%, methane 30%, petroleum 25%. Solid paraffin: 3%.

Synthetic petroleum obtained from bituminous coals: is characterized by the following group hydrocarbon composition (total content of substances of different classes in all fractions):

Aromatic hydrocarbons 30%, methane 50%, petroleum 20%. Solid paraffins: 5%.



Synthetic petroleum obtained from hard coals and anthracites is characterized by the following group hydrocarbon composition (total content of material of different classes in all fractions):

Aromatic hydrocarbons 50%, methane 20%, naphthene 30%. Solid paraffins: 2%.

Physical-chemical characteristics of the synthetic fuel oil obtained.

A fuel oil analogous to M-100 fuel oil is obtained: yield: 90% of the organic raw material.

Carbon content 86%, hydrogen 12%, sulfur 1.5%, nitrogen and oxygen no more than 0.5%.

Density at 20°C - 0.950-0.990

Viscosity of the VU condition - 15.5 (80°C)

Ash content - 0.30-1.0%

Sulfur no more than 2% - (on the average 1-1.5%)

hardening temperature- +25°C

flash temperature - +125°C

Combustion heat - 9500 kcal/kg

pitches - 12-15%

asphaltenes - 0.5-1.5%

carbenes and carboids - 1.3%

excise pitches - 28%

coke - 10-15%

The synthetic fuel oil obtained from peats is characterized by the following group hydrocarbon composition:

Aromatic hydrocarbons 45%, methane-naphthene hydrocarbons 55%.

The synthetic fuel oil, obtained from brown coals, is

characterized by the following group hydrocarbon composition.

Aromatic hydrocarbons 40%, methane-naphthene hydrocarbons 60%.

Synthetic fuel oil obtained from hard coals and anthracites is characterized by the following group hydrocarbon composition:

Aromatic hydrocarbons 60%, methane-naphthene hydrocarbons 40%.

Physical-chemical characteristics of the tars obtained:

Yield: 90% of the organic weight of raw material

- peat tar:

density - 0.95-1.05 g/cm<sup>3</sup>

viscosity - 10 cSt (50°C)

sulfur - 9.5%

combustion heat - 8700-9000 kcal/kg.

Hydrocarbons around 40% including aromatic, saturated and unsaturated:

phenol - 20%

solid paraffin - 3.0-8.0%

Oxygen compounds around 40% including:

acids and phenols - 10%

asphaltenes 0-2% - brown coal tar:

density - 0.9-1.00 g/cm<sup>3</sup>

viscosity - 5 cSt (50°C)

sulfur 0.5%

combustion heat - 9900-10000 kcal/kg.

Hydrocarbons around 40%, among them aromatic, saturated and unsaturated:

phenols - 8%

solid paraffin - 7.0-18%

oxygen compounds around 16%, including:

acids and phenols - 10%

asphaltenes - up to 5% - hard coal tar:

density - 0.96-1.08 g/cm<sup>3</sup>

viscosity - 15 cSt (50°C)

sulfur 1.5%

combustion heat - 9400 kcal/kg.

Hydrocarbons around 40%, among them aromatic, saturated, and unsaturated

phenols - 15%

solid paraffin - 4-6%

Oxygen compounds around 30%, including:

acids and phenols - 10%

asphaltenes up to 30% - shale tar:

density - 0.95-1.00 g/cm<sup>3</sup>

viscosity - 10 cSt (50°C)

sulfur 0.9%

combustion heat - 9800 kcal/kg.

Hydrocarbons around 40%, among them aromatic, saturated and unsaturated

phenols - 15%

solid paraffin - 0-1%

Oxygen compounds around 30%, including:

phenols - 15%

solid paraffin - 0.1%

oxygen compounds around 30%, including:

acids and phenols - 10%

asphaltenes up to 15%

Determination method:

The carbon and hydrogen content of coals and in the hydrocarbons obtained (in synthetic petroleum, in fuel oil and in tar) was determined by the spectral method and the method of burning a weighed amount in an oxygen flow - "pyrolytic burning".

The sulfur content in the petroleum investigated was determined by the method of burning in a quartz tube, in fuel oil and tar - burning in a tube oven (GOST 1437-56) and burning in a calorimetric bomb (GOST 3877-49). The mercaptan and hydrogen sulfide content of the petroleum was determined by the potentiometric method. The nitrogen content of the petroleum was determined by the Dumas method.

The pitch-asphalt substance content was determined by the adsorption method (adsorption on silica gel). The composition of the pitch-asphalt substances was determined by Markusson analysis. The asphalt content was determined by the Gold "cold method". The density of the artificial petroleum was determined by the pycnometric method on a pycnometer, the viscosity - by the method of determining the conditional viscosity, with a VU type viscometer. The molecular weight of the pitches was determined on a mass spectrometer and by the cryoscopic method. The density of the pitches was determined by the pycnometric method. The sulfur content of the pitch-asphalt substances was determined by the method of burning in a quartz tube, the nitrogen content by the Dumas method, the oxygen content was determined (calculated) according to the element difference, and also was determined in analyzers of the magnetic and polarization type.

Formulation of Invention:

1. A microbiological method of producing petroleum hydrocarbons and individual hydrocarbon fractions of solid fossil fuels, wherein a suspension of solid fossil fuels subjected to grinding is treated with cultures of *Thiobacillus aquaesulis*, or *Thiobacillus denitrificans*, or *Thiosphaera pantotropha* bacteria, taken in any combinations, in the presence of a compound that is a hydrogen donor.

2. The method according to paragraph 1, wherein the process is conducted at 4-60°C preferably at 18-24°C, and at pH 6-9.

3. The method according to paragraph 1, wherein humites, hard and brown coals, and peat are used as the raw material

4. The method according to paragraph 1, wherein acids, phenols, naphthalene, tetrahydronaphthalene, and cresol are used as hydrogen donor compounds.

5. The method according to paragraphs 1-4, wherein peat is used as the raw material, and the above-mentioned cultures of bacteria are used together with bacteria of the species *Thiobacillus thioparus*.

## Degradation of Coal by the Fungi *Polyporus versicolor* and *Poria monticola*

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We report that two species of basidiomycete fungi (*Polyporus versicolor* and *Poria monticola*) grow in minimal liquid or solid medium when supplemented with crushed lignite coal. The fungi also grow directly on crushed lignite coal. The growth of both fungi was observed qualitatively as the production and extension of hyphae. No fungal growth occurred in minimal agar medium without coal. The fungi degraded solid lignite coal to a black liquid product which never appeared in cultures unless fungi and coal were present together. Apparently, lignite coal can serve as the principal substrate for the growth of the fungi. Infrared analyses of the liquid products of lignite degradation showed both similarities to and differences from the original lignite.

Although coal conversion technology has been studied since at least 1780 (9), little attention has been directed toward the biological degradation of coal, perhaps because there have been few reports of organisms which can metabolize coal. Belly and Brock (4) developed a technique for measuring <sup>14</sup>CO<sub>2</sub> uptake by chemolithotropic bacteria directly in pyritic materials associated with coal and coal refuse. Maximal <sup>14</sup>CO<sub>2</sub> uptake occurred in coal refuse material 2 to 3 years old. Only slight incorporation was reported for fresh material or material 40 years old. Kucher and Turovskii (13) reported optimal conditions for the growth of the yeast *Candida tropicalis* in aqueous extracts of oxidized coal. Holladay et al. (10) reported the biodegradation of phenolic waste liquors resulting from the pyrolysis of coal.

We found no reports of fungi that grow directly on and metabolize naturally occurring coal. This is the first report of fungal growth either directly on lignite coal or in minimal medium supplemented with crushed coal.

### MATERIALS AND METHODS

*Polyporus versicolor* ATCC 12679 and *Poria monticola* ATCC 11538 were obtained from the American Type Culture Collection, Rockville, Md. Lignite coal was obtained from the American Colloid Co., Skran-ton, N. Dak. A geological analysis of the lignite indicated that it originated in the upper Paleocene era. All coal used in the experiments was derived from bulk pieces in which the grain of the woody tissues from which it was formed was preserved.

Both *Polyporus versicolor* and *Poria monticola* were routinely maintained and cultured in Sabouraud maltose broth and on Sabouraud maltose agar (28°C; 80% relative humidity; pH 5.8). All culture media were

sterilized in 1-pint (ca. 0.473-liter) jars before inoculation, all fungal transfers were performed aseptically in a laminar flow hood, and all materials which came into contact with the cultures were sterilized before use. Experimental cultures were incubated as described for stock cultures. Two-week-old 1-pint broth cultures of both *Polyporus* and *Poria* mycelia were aseptically accumulated and placed into 100-ml milk dilution bottles containing sterile glass beads and distilled and deionized (D/D) water. The bottles were then stoppered and shaken to disrupt hyphae. Accumulated hyphal suspensions were then filtered through ground-glass filter paper and washed with 1 liter of D/D water to eliminate residual broth media. Approximately 0.5 g (wet weight) of mycelium was then transferred to Noble agar medium (1% [wt/vol] Noble agar, D/D water) and lignite agar medium (1% [wt/vol] Noble agar, 7% [wt/vol] powdered lignite, D/D water; lignite powder was added to the warm dissolved agar, and the pH was adjusted to 5.8 with 0.5 N KOH). Before inoculating crushed lignite substrate medium or solid lignite substrate medium, the hyphae were filtered as described above, and 0.5-g samples were suspended in 1 ml of D/D water. To prepare crushed lignite substrate medium, powdered lignite was filtered through an 80/100-mesh screen and hydrated with 0.1 N KOH to 130% of its water-holding capacity (1); the pH was adjusted to 5.8. Solid lignite substrate medium was prepared as follows. Sabouraud maltose agar (3 to 6%, wt/vol) was inoculated with 1-ml samples of the hyphal suspension described above, and the hyphae were spread over the surface with a sterile glass rod. These cultures were then incubated for 5 days. A sterilized polypropylene grid was placed on top of each culture, and a small (2-cm<sup>3</sup>) sterile piece of solid lignite coal was placed on top of the grid to isolate the coal from contact with the agar. The cultures were then incubated for 28 days.

Infrared (IR) analyses of materials were done on a Perkin-Elmer infrared double-beam grating spectrophotometer (model 283). Crushed lignite was analyzed

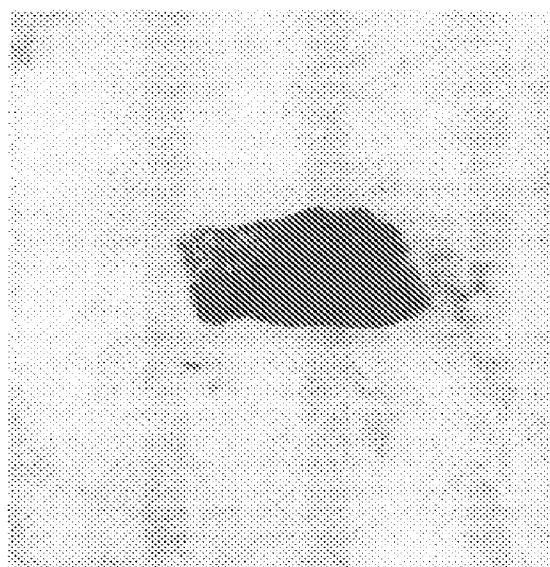


FIG. 1. Early stage of growth of *Polyporus* hyphae onto a piece of solid lignite coal 10 days after the lignite piece was placed onto a polypropylene grid which separates the lignite from the surface of the culture medium.

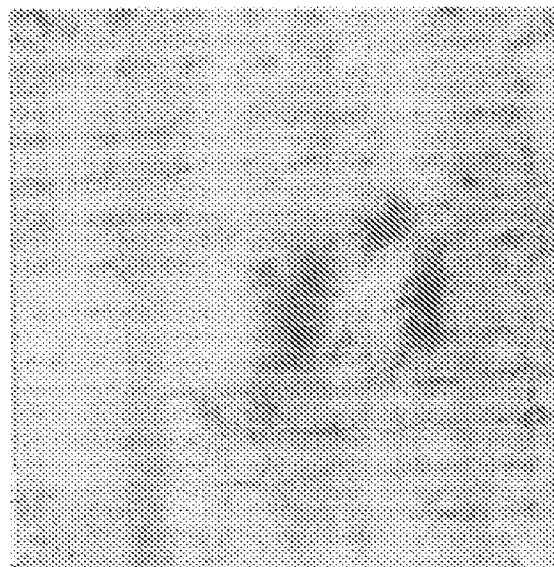


FIG. 2. Growth of *Polyporus* hyphae onto a piece of solid lignite coal 28 days after the lignite piece was placed onto a polypropylene grid which separates the lignite from the surface of the culture medium. Note that the hyphae have completely covered the lignite piece.

as a mugol mull cast film on both sodium chloride and silver chloride window materials. A hexachloro-1,3-butadiene mull was prepared for the hydrocarbon regions 3,000 to 2,800, 1,500 to 1,300, and 750 to 650  $\text{cm}^{-1}$ . IR analyses of liquid degradation products were performed as cast films on sodium chloride and silver chloride window materials. Cast films were both air and oven dried (120°F; 60 min) before running the spectra. No differences were observed between these preparations.

## RESULTS

Rapid growth of both *Polyporus versicolor* and *Poria monticola*, measured qualitatively as the production and extension of hyphae, occurred on the Sabouraud malt agar used routinely to maintain the fungi. The agar in these continuous cultures always maintained the original straw color.

The growth of *Polyporus versicolor* also occurred in lignite agar medium and on crushed lignite coal. Hyphal growth was more extensive and luxurious on lignite agar than on crushed lignite substrate. The production and growth of *Poria* hyphae were less extensive than those of *Polyporus* hyphae in identical cultures of lignite agar. No fungal growth was observed in Noble agar cultures, and the agar maintained the original pale straw color. The agar may be a source of micronutrients which enhance fungal growth, but Noble agar alone cannot maintain the fungi or allow their growth. We interpreted this to mean that the principal substrate for the growth of both *Polyporus versicolor* and *Poria monticola* in lignite agar and on crushed lignite sub-

strate was the lignite coal. In the solid lignite cultures, vegetative hyphae of both fungi were observed to grow up onto the solid lignite piece and completely cover it (Fig. 1 and 2). The grain of the plant material from which the lignite was formed could be seen on the pieces, and the hyphae followed the pattern of the grain. In approximately half of all cultures, several drops of a black viscous liquid substance appeared on top of the hypha-covered lignite pieces (Fig. 3 and 4). After some of this liquid was drawn off for analysis, several more milliliters of black liquid were produced. The production appeared to be continuous because the Sabouraud malt agar, initially a pale straw color, progressively darkened and became uniformly black during the incubation period after the fungal hyphae covered the lignite piece. In identical cultures incubated as described above but without inoculation with *Polyporus versicolor* or *Poria monticola* and in Sabouraud maltose agar cultures of the fungi, the agar maintained a pale straw color and never darkened during the time of testing (Fig. 5). This established that the source of the black liquid was the lignite coal and that the digestion of the coal by *Polyporus versicolor* or *Poria monticola* was necessary to produce the black liquid digestion product.

Both lignite and the black liquid exudates were analyzed by IR spectroscopy. The absorption spectra are shown in Fig. 6. Figure 6A shows the IR absorption spectrum of a mugol mull of similar lignite coal samples from which

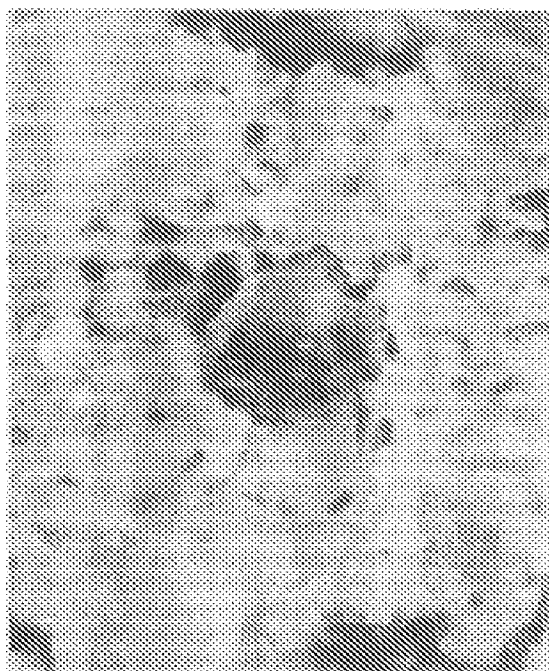


FIG. 3. Production by *Polyporus versicolor* of a black liquid digestion product from a solid piece of lignite treated as described in the legend to Fig. 2. The black liquid appears as several drops on top of the hypha-covered lignite piece and has also diffused into the medium, causing it to darken.

the black liquid digestion product was produced. The band at  $3,400\text{ cm}^{-1}$  (H-bonded OH) is broad due to extensive hydrogen bonding (7). In lignite, the bands at  $2,920$  and  $2,850\text{ cm}^{-1}$  (aliphatic C—H stretching) do not appear to indicate a high degree of aliphatic hydrocarbon structure (4, 8). This is typical of lignite coal. The shape and intensity of the band at  $1,600\text{ cm}^{-1}$  are typical of lignite, and a controversy over specific band assignment has been reported (8). The shoulder at  $1,700\text{ cm}^{-1}$  and the band at  $1,600\text{ cm}^{-1}$  combined probably represent high concentrations of either conjugated aromatic structures or chelated and conjugated carbonyl structures, such as acetylacetone, kojic acid, or hydroxyacetophenes, or both (3, 8). The remaining regions of complex absorptions from  $1,300$  to  $900\text{ cm}^{-1}$  represent combinations of phenoxy and ether linkages as well as clay minerals ( $1,040$  to  $910\text{ cm}^{-1}$ ) (7). The lack of absorption peaks at  $720$  to  $800\text{ cm}^{-1}$  indicates that no hydrocarbon chains longer than  $\text{C}_4$  were present (3).

Figures 6B and C show IR absorption spectra of the black viscous liquid exudates produced from fungus-covered lignite pieces in cultures with *Polyporus versicolor* and *Poria monticola*, respectively. Both spectra show similar absorption patterns in the regions of  $3,400$  and  $1,600\text{ cm}^{-1}$ . These also represent the principal functional group absorptions of lignite (4, 7, 8). The

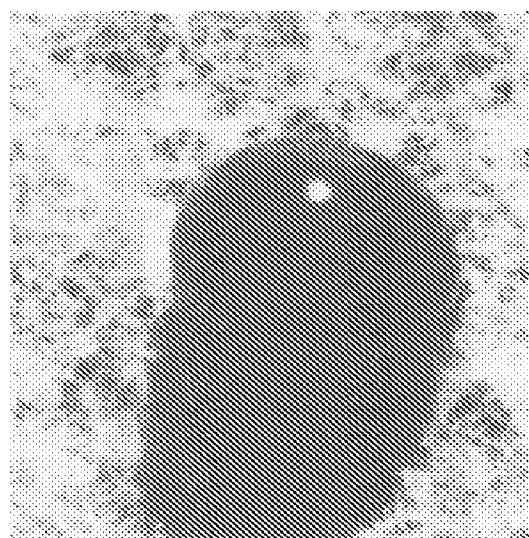


FIG. 4. Close-up of a drop of the black liquid digestion product produced as described in the legend to Fig. 3 on the surface of the lignite piece. Note the arrangement of fungal hyphae on the lignite and the spherical nature of the liquid product.

spectrum in Fig. 6B also shows functional group absorptions at  $3,200\text{ cm}^{-1}$  (N—H) (14),  $1,720\text{ cm}^{-1}$  (C=O) (3),  $1,680\text{ cm}^{-1}$  (aryl ketones or conjugated carbonyl) (5), and  $1,410\text{ cm}^{-1}$  (carboxylic anionic groups, carbonate, and  $\text{CH}_2$ ) (4).

The spectra in Fig. 6B and C also show a shift of the band at  $1,600\text{ cm}^{-1}$  present in lignite down to  $1,580\text{ cm}^{-1}$ . Band shifts to lower wave numbers generally represent an increase in the resonance of functional groups (8). This same shift of absorption has been reported to occur in the IR absorption spectrum of lignite after pyrolysis at  $450^\circ\text{C}$  (6). We interpreted this result to mean that conjugated aromatic rings responsible for absorption at  $1,600\text{ cm}^{-1}$  had been structurally modified by the digestion of lignite by *Polyporus versicolor* and *Poria monticola* at  $28^\circ\text{C}$  in a manner similar to the ring modification produced by pyrolysis at  $450^\circ\text{C}$ .

The spectrum in Fig. 6C shows fewer regions of IR absorption compared with the spectrum in Fig. 6B; this indicates the presence of fewer functional groups.

## DISCUSSION

Lignite coals originate from the compaction and induration of the altered remains of diverse plants, all of which contain lignin in various amounts. During the coalification process, lignin-like structures are preserved and may predominate over other woody tissue remains. Buravis et al. (6) reported that the chemical analysis of young lignites showed that they contain from 35 to 70% lignin-like compounds and that older lignites are likely to contain compounds formed



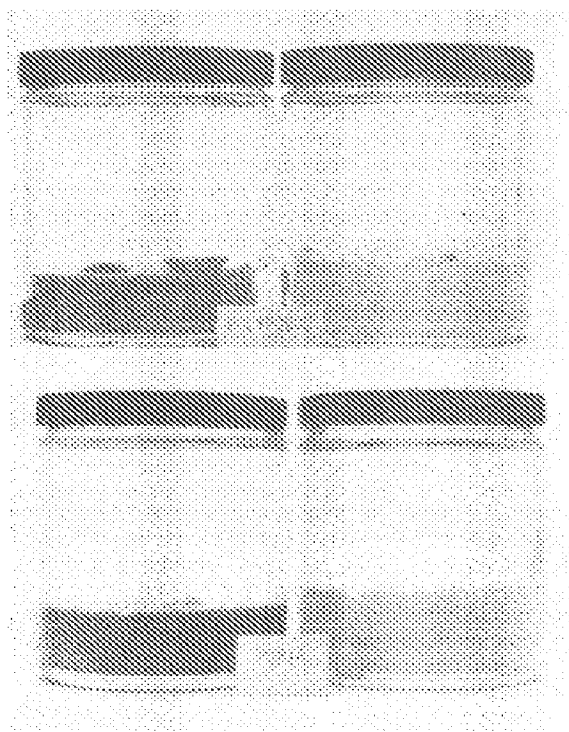


FIG. 5. Darkening of the Sabouraud maltose agar culture medium by *Polyporus versicolor* (top left) and *Poria monticola* (bottom left) after the production of the black liquid digestion product from lignite. In identical cultures incubated as described in the text but without the coal, the medium remained a pale straw color (right).

by the condensation of these structures. Thus, lignin and lignite share a common origin, each being derived from plant tissue.

*Polyporus versicolor* and *Poria monticola* are wood-decaying organisms with two different mechanisms of digestive action. *Polyporus versicolor* decomposes lignified tissues by digesting polyphenylpropane polymers and associated aromatic ring structures of the lignin polymer. This white-rot decay is reported to be based on the production of polyphenoloxidase and peroxidase enzymes (15, 16). Ander et al. (2) have reported that a phenol oxidaseless mutant of the white-rot fungus *Sporotrichum pulverulentum* lost the ability to degrade lignin and other wood components. The enzyme cellobiose:quinone oxidoreductase has been reported in cultures of *Polyporus versicolor* and seems to be important in lignin degradation (2). However, the mechanism and biochemical pathways of lignin biodegradation have not been specifically defined (12). The polymeric structures derived from lignin and present in lignites might serve as suitable substrates for digestion by *Polyporus versicolor*.

*Poria monticola* decomposes wood by digesting polysaccharides, producing only a small loss of lignin. This brown-rot decay has been report-

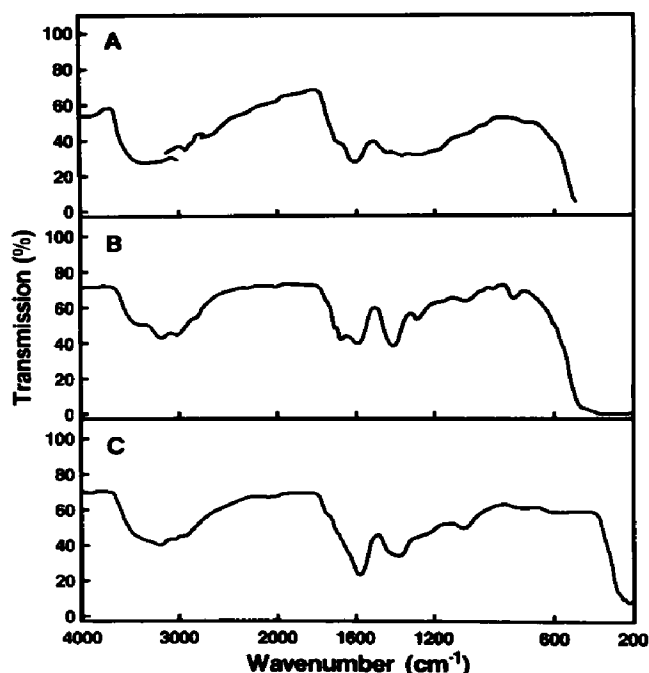


FIG. 6. IR absorption spectra of a mugol mull of lignite (A) and the black liquid digestion products produced from lignite by *Polyporus versicolor* (B) and *Poria monticola* (C). Note the shift of the characteristic band at  $1,600\text{ cm}^{-1}$  of lignite to bands at  $1,590\text{ cm}^{-1}$  in both of the liquid digestion products.

ed to be based on the production of  $\beta$ -glucosidases which attack principally celluloses (11). Recently, Schmidt et al. (17) have proposed that the brown-rot mechanism occurs nonenzymatically through the production of free radicals which degrade cellulosic polymers. This physiological difference could explain why the growth of *Polyporus versicolor* was more extensive and rapid than that of *Poria monticola* on lignite agar medium.

We have not established that the degradation of lignite occurred enzymatically or that specific digestive enzymes were produced. Although some activity of fungal growth was necessary to degrade the lignite, the biochemical mechanism has not been defined.

The fact that only half of all cultures on lignite pieces produced the black liquid digestion product may indicate that a process of adaptation to lignite has occurred in these cultures or that the lignite sample has a heterogeneous chemical composition. Due to differences in the digestive action of the two fungi, variations in the chemical composition of the black liquid breakdown products of lignite would be expected; this was confirmed by IR analyses. The products produced from lignite by *Polyporus versicolor* and *Poria monticola* have similarities in basic composition to lignite and to each other, but they appear to be different.

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## TECH.SIGHT

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## Techwire

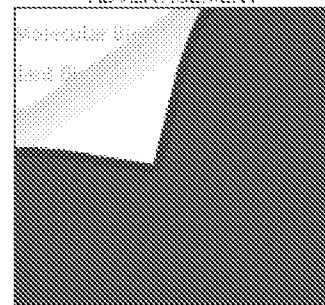
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Traditional biochemical techniques such as protein purification are powerful tools for identifying proteins on the basis of their function. Yet, isolating the genes that encode these proteins is often cumbersome, requiring multiple steps of purification, peptide sequencing, and subsequent isolation of complementary DNA (cDNA) [HN1]. An alternative approach to functional gene identification, which perhaps could be called "reverse" biochemistry, is expression cloning, in which cDNAs are first translated in prokaryotic or eukaryotic cells and then assayed for a specific biologic activity. This approach circumvents the requirement for protein purification, yet it retains the use of a functional assay as the basis of cDNA identification. Cell-based expression cloning strategies, which use mammalian cell lines or *Xenopus laevis* oocytes to express encoded proteins, have identified genes encoding membrane receptors, transmembrane channels, and secreted growth factors, but they have not been widely used to identify intracellular activities. Here we discuss a new cell-free expression cloning approach (1) that substantially expands the range of biochemical assays that can be used to identify a cDNA on the basis of its function.

The cloning strategy is straightforward [see figure and (2, 3) for details]. Rather than expressing cDNAs in intact cells, where the encoded proteins are not freely accessible, small plasmid pools (~50 to 100 clones per pool) are instead expressed in vitro in a single step by means of a coupled transcription-translation system (the Promega TNT system) [HN2]. Although this system is optimized to express single proteins, we found, surprisingly, that about 30 proteins can be expressed in a single reaction. Each protein pool is then screened for the presence of a desired biochemical activity, and positive pools are progressively subdivided until a single cDNA encoding the active protein is isolated. In principle, in vitro expression cloning (IVEC) can be used to rapidly identify cDNAs on the basis of almost any biochemical property for which an assay can be developed.

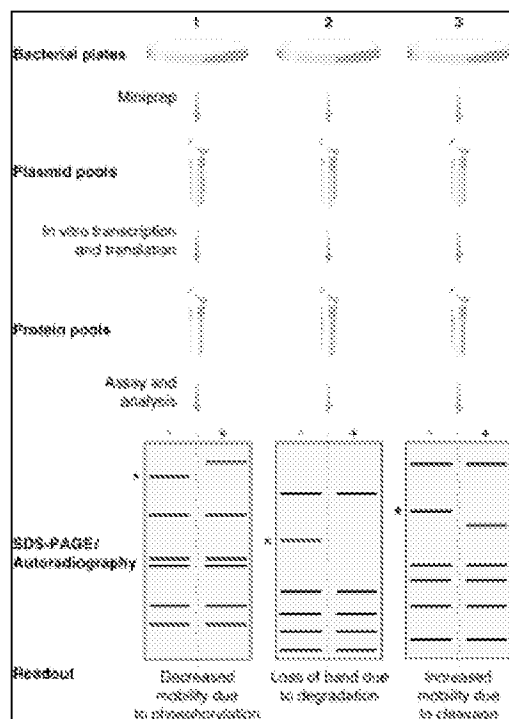
Many cellular processes are regulated by posttranslational protein modification, yet there are no general techniques that can identify the substrates of modifying enzymes on the basis of this property. By screening small pools of radiolabeled proteins, we have been able to systematically identify substrates of enzymes such as kinases and proteases. In a typical assay, a kinase or

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protease activity, either present in a crude extract or in purified form, is incubated with a radiolabeled protein pool, which is then resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; see figure) [HN3]. Because posttranslational modifications such as phosphorylation or proteolysis often change the electrophoretic mobility of a protein, pools containing a candidate substrate are identified by scoring for proteins whose mobility is altered by incubation with a given enzyme.



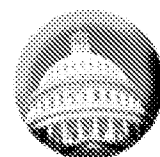
**The strategy of in vitro expression cloning.** An unamplified cDNA expression library [HN13] is plated at a density of approximately 100 clones per bacterial plate. Pooled plasmid DNA is obtained by scraping colonies from each plate and performing a small-scale plasmid purification. Each plasmid pool is then transcribed and translated in vitro with a commercially available system, such as the TNT system from Promega. The resulting protein pool is then assayed for the presence of an activity. In the illustrated experiment, a radioactive amino acid is included in the translation system to specifically label the pool of proteins. Incubation of a pool with a modifying enzyme (lanes labeled +) such as a protease or kinase can result in a change in mobility of a substrate (bands marked with asterisk). Pool 1 contains a protein whose mobility is reduced following treatment with a kinase; Pool 2 contains a protein that is degraded following treatment with an extract containing an activated proteolytic system; Pool 3 contains a protein that is specifically cleaved following treatment with a protease, decreasing its apparent molecular mass. Once a pool containing a candidate activity is identified, the original cDNA pool is subdivided and retested until the single cDNA encoding the protein of interest is isolated.

We applied this approach to identify proteins that become phosphorylated during mitosis (3). Pools of radiolabeled proteins were incubated with either *Xenopus* [HN4] interphase or mitotic extracts, and candidate substrates were identified by screening for proteins whose electrophoretic mobility was reduced by treatment with the mitotic extract (panel 1 of figure). In a complementary screen, a monoclonal antibody [HN5] that recognizes mitotic phosphoproteins was used to identify substrates that could be immunoprecipitated only after treatment with mitotic extract. After screening 12,000 proteins, we identified 20 distinct mitosis-specific phosphoproteins, including five that share no sequence similarity with known proteins.

We developed a similar functional screen to identify proteins that are degraded by the ubiquitin

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system [HN6] as cells exit mitosis (2, 4). Candidate substrates were identified by screening pools for proteins that were degraded following incubation in a *Xenopus* mitotic egg extract (panel 2 of the figure). In addition to identifying several forms of cyclin B, as expected, this screen has identified a novel protein that shares little sequence similarity with cyclin B, with the exception of a small motif that is required for ubiquitin-dependent degradation.

Proteolysis of a different sort regulates the process of apoptosis [HN7], or programmed cell death. To screen for substrates that are specifically cleaved during apoptosis, pools of labeled proteins were incubated with extracts derived from apoptotic cells, and assayed for the disappearance of a labeled band or the appearance of a proteolytic fragment (2, 5) (panel 3 of figure). In a similar screen for caspase substrates, purified proteases have been used in lieu of apoptotic extracts (6). These approaches have identified several candidate substrates whose roles in executing apoptosis are now being studied.

Although IVEC has successfully identified proteins that are posttranslationally modified, its potential applications are much broader, for it can be employed in screens that detect physical interactions between pool proteins and a wide variety of "bait" molecules. For example, to screen for transcription factors capable of binding specific DNA elements, unlabeled protein pools were incubated with a labeled DNA fragment and then assayed for protein-DNA binding by use of a gel mobility shift assay (2, 7). Although this method has only been used to identify proteins that bind to "naked" DNA, in principle the bait could be a preassembled complex of DNA bound to known transcription factors. RNA-binding proteins could be isolated in a similar fashion.

Direct protein-protein interactions can also be used as a basis for cDNA identification. To identify binding partners, for example, a bait protein could be covalently coupled to a bead, mixed with radiolabeled protein pools, and interacting proteins reisolated by centrifugation. An important advantage of IVEC over conventional two-hybrid screening [HN8] is that protein pools can be modified with purified enzymes or cellular extracts before the affinity screen is conducted. Another advantage is that the bait could be a large complex (such as a multimeric enzyme), a protein machine (such as a ribosome), or even a virus or cell organelle.

By using an antibody as a bait, IVEC may provide a useful alternative to traditional  $\lambda$ -gt11 expression cloning [HN9]. Immunoprecipitation-based assays may be especially useful when an antibody recognizes only the native form of a protein, or when the protein must be modified to be recognized. For example, a screen could be designed to identify tyrosine-phosphorylated proteins recognized by antiphosphotyrosine antibodies.

Many complex cellular processes can now be reconstituted in vitro, extending the capabilities of IVEC beyond simple interaction-based screens. The process of secretion can be modeled in vitro by supplementing the translation system with purified microsomes. A screen for secreted proteins could therefore be developed, either by using a protease protection assay, or by scoring for changes in electrophoretic mobility that occur as a consequence of the microsome-dependent cleavage of a signal sequence. The incorporation of an epitope or chemical tag in the expressed proteins could also enable the design of assays that identify proteins on the basis of localization to other cellular structures. Pools of biotinylated proteins [HN10] could be incubated with complex cellular extracts, and incorporation into nuclei or centrosomes could be monitored with streptavidin-coupled fluorophores [HN10] and microscopy.

Enzyme-activity-based screens are another unexplored but potentially exciting application of IVEC. The assays would be similar to those used in standard biochemical approaches, with the exception that protein pools rather than chromatographic fractions would be tested for activity. For example, protease cDNAs could be cloned by testing unlabeled protein pools for the ability to cleave a known substrate. This approach may also expedite the identification of new "extremophilic" enzymes that operate at extremes of temperature, pH, or ionic strength.

Enzyme activity screens could also be used to identify mutant enzymes with novel properties. To identify mutant enzymes that are insensitive to an inhibitor, small pools of mutagenized proteases could be constructed and screened for activity in the presence of the inhibitor. This approach may facilitate the identification of mutations that lead to drug resistance, or, using sequential rounds of mutagenesis and screening, enable investigators to evolve enzymes with activity toward novel substrates.

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Although IVEC dramatically expands the types of assays that can be used as a basis for expression cloning, it has several limitations. First, IVEC is unlikely to succeed when multiple proteins are required for activity, as the likelihood of expressing multiple subunits in the same pool is low. If the putative binding partners are known, it may be possible to supply them as recombinant proteins or fractionated extracts. Second, for activity-based approaches to be successful, the expression system must be devoid of the activity being assayed. We have only used reticulocyte lysates to express cDNA pools; alternative expression systems, such as wheat-germ lysates, may have different background activities. Finally, the concentration of expressed proteins is low relative to the protein concentration of the lysate. Although protein labeling facilitates the identification of substrates, the detection of enzymatic or binding activities may require the expression of greater amounts of protein. The use of smaller cDNA pools increases the abundance of each protein, but also makes the screening process more time-consuming. Alternatively, it may be possible to isolate and concentrate the expressed proteins, either by constructing epitope-tagged cDNA libraries or by incorporating biotinylated amino acids to specifically tag newly expressed proteins.

IVEC is feasible in small laboratories because DNA preparation and protein expression technologies have become rapid and reproducible. The initiation of saturating, genome-wide screening, however, will depend on automating the process. Fortunately, the same robotic systems that have been developed for genome sequencing [HN11] and high-throughput drug screening should be easily adaptable to the steps of cDNA pool preparation and in vitro translation, and, in many cases, to the biochemical assay itself. In the not-so-distant future, the availability of completely normalized libraries, in which each cDNA found within the genome is represented once, will provide the ultimate resource for IVEC. This is currently feasible in *Saccharomyces cerevisiae*, in which the sequence of each of its ~6200 potential genes is known. These cDNAs could be expressed and tested individually, or pooled together to expedite assays.

IVEC is a hybrid technique, containing elements of both genetics and biochemistry, that has enabled us to ask new questions that could not be addressed with either approach alone. Whereas cell-based expression cloning technologies have excelled in the identification of secreted growth factors and their receptors, IVEC now makes it possible to identify proteins that are active in the cytoplasm and nucleus. Within the coming decade, the human genome will be completely sequenced. Expression cloning techniques, using both cell-based and in vitro expression systems, will be important tools for characterizing the tens of thousands of genes whose sequences will be available, yet whose functions remain obscure.

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## HyperNotes

### Related Resources on the World Wide Web

#### NUMBERED HYPERNOTES

1. The Institute for Genomic Research provides on the Web an extensive database of human cDNAs. You can query the database for individual sequence tagged site (STS) reports, for transcripts with mapped STSs, and for chromosomes with mapped transcripts.
2. This section of the Promega Web site provides the entire technical manual on the TNT quick coupled transcription/translation system. The Frequently Asked Question Section features answers to the most common questions about this coupled reticulocyte lysate system.
3. E. Rybicki and M. Purves of the department of microbiology at the University of Cape Town (South Africa) present this online molecular biology techniques manual that provides a description of the SDS-PAGE technique and gives the protocols for running such gels.
4. This Web site provides stocks of *Xenopus* frogs. Besides viewing the online pricing catalog, readers can also access a series of links about *Xenopus laevis*. One such link well worth visiting is the *Xenopus* Molecular Marker Resource maintained by the Vize lab at the University of Texas, Austin. Besides featuring an extensive index, this site features lab protocols and many pictures of developing embryos stained with specific markers.
5. The Hybridoma Databank maintained by the American Type Culture Collection is a collection of records characterizing monoclonal antibodies and hybridomas. The current database contains approximately 26,000 records.
6. This one-page online summary about ubiquitin includes a picture of its tertiary structure and references.
7. D. Davies of the Fluorescence Activated Cell Sorter Laboratory (FACS) of the Imperial Cancer Research Fund provides this description of the various laboratory methods used to study apoptosis, including propidium iodide staining, strand break labeling, and annexin V staining. The descriptions are enhanced with pictures. Additional apoptosis resources have been reviewed by Medsite Navigator and cover methods, journals, and suppliers of kits.
8. The Brent lab in the department of genetics at The Massachusetts General Hospital and Harvard Medical School provides this description of the two-hybrid method and the various protocols available. A significant number of the files need to be downloaded and viewed with a text editor.
9. This site, whose space is provided by Anderson Unicorn Group, features an impressive database of molecular biology vectors such as phages, phagemids, plasmids, cosmids, viruses, and YACs. You can find specific information about l-gt11 as well.
10. Streptavidin/Biotin systems are available from several suppliers such as Amersham, Biomedex and Biotrend. Prozyme, a supplier of streptavidin-biotin systems, features product description and pricing.
11. A review of robotic systems for sequencing the human genome was published in the 20 June

issue of Tech.Sight. Readers can also participate in an online discussion forum about this topic.

12. The U.S. Patent and Trademark Office site features a searchable database of U.S. patents, forms to apply for patents, information on patent fees and review procedures, as well as legal information. The viewer should start with the site map to try to get oriented to this Web site full of useful information.
13. Research Genetics, a supplier of human DNA libraries, allows online access to its catalog for cDNAs with descriptions and pricing. Customers can even order libraries online. Another supplier of cDNA libraries is Genome Systems, whose catalog is also available online.

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# Microdiesel: *Escherichia coli* engineered for fuel production

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Biodiesel is an alternative energy source and a substitute for petroleum-based diesel fuel. It is produced from renewable biomass by transesterification of triacylglycerols from plant oils, yielding monoalkyl esters of long-chain fatty acids with short-chain alcohols such as fatty acid methyl esters and fatty acid ethyl esters (FAEEs). Despite numerous environmental benefits, a broader use of biodiesel is hampered by the extensive acreage required for sufficient production of oilseed crops. Therefore, processes are urgently needed to enable biodiesel production from more readily available bulk plant materials like sugars or cellulose. Toward this goal, the authors established biosynthesis of biodiesel-adequate FAEEs, referred to as Microdiesel, in metabolically engineered *Escherichia coli*. This was achieved by heterologous expression in *E. coli* of the *Zymomonas mobilis* pyruvate decarboxylase and alcohol dehydrogenase and the unspecific acyltransferase from *Acinetobacter baylyi* strain ADP1. By this approach, ethanol formation was combined with subsequent esterification of the ethanol with the acyl moieties of coenzyme A thioesters of fatty acids if the cells were cultivated under aerobic conditions in the presence of glucose and oleic acid. Ethyl oleate was the major constituent of these FAEEs, with minor amounts of ethyl palmitate and ethyl palmitoleate. FAEE concentrations of  $1.28 \text{ g l}^{-1}$  and a FAEE content of the cells of 26 % of the cellular dry mass were achieved by fed-batch fermentation using renewable carbon sources. This novel approach might pave the way for industrial production of biodiesel equivalents from renewable resources by employing engineered micro-organisms, enabling a broader use of biodiesel-like fuels in the future.

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## INTRODUCTION

A major challenge mankind is facing in this century is the gradual and inescapable exhaustion of the earth's fossil energy resources. The combustion of those fossil energy materials lavishly used as heating or transportation fuel is one of the key factors responsible for global warming due to large-scale carbon dioxide emissions. In addition, local environmental pollution is caused. Thus, alternative energy sources based on sustainable, regenerative and ecologically friendly processes are urgently needed.

One of the most prominent alternative energy resources, attracting more and more interest in recent years with the price for crude oil reaching record heights, is biodiesel, which is a possible substitute for petroleum-based diesel fuel. Biodiesel is made from renewable biomass mainly by alkali-catalysed transesterification of triacylglycerols (TAGs)

from plant oils (Ma & Hanna, 1999). It consists of monoalkyl esters of long-chain fatty acids with short-chain alcohols, primarily methanol and ethanol, resulting in fatty acid methyl esters (FAMEs) and fatty acid ethyl esters (FAEEs). Biodiesel offers a number of interesting and attractive beneficial properties compared to conventional petroleum-based diesel (for an overview see Krawczyk, 1996). Most important, the use of biodiesel maintains a balanced carbon dioxide cycle since it is based on renewable biological materials. Additional environmental benefits are reduced emissions (carbon monoxide, sulphur, aromatic hydrocarbons, soot particles) during combustion. Biodiesel is non-toxic and completely biodegradable. Due to its high flash point, it is of low flammability and thus its use is very safe and non-hazardous. Furthermore, it provides good lubrication properties, thereby reducing wear and tear on engines. Pure biodiesel or biodiesel mixed in any ratio with petroleum-based diesel can be used in conventional diesel engines with no or only marginal modifications, and it can be distributed using the existing infrastructure. Biodiesel is already produced in a growing number of countries on a large scale (e.g. 1 080 000 t biodiesel was produced in Germany in 2004: Bockey & von Schenck, 2005).

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**Abbreviations:** FAEE, fatty acid ethyl ester; FAME, fatty acid methyl ester; TAG, triacylglycerol; WS/DGAT, wax ester synthase/acyl-coenzyme A:diacylglycerol acyltransferase.

Despite these positive ecological aspects, however, biodiesel, as currently produced on a technical scale, has also numerous drawbacks and limitations. (1) Production is dependent on the availability of sufficient vegetable oil feedstocks, mainly rapeseed in Continental Europe, soybean in North America and palm oil in South East Asia. Therefore, industrial-scale biodiesel production will remain geographically and seasonally restricted to oilseed-producing areas. (2) Vegetable oils predominantly consisting of TAGs can not be used directly as diesel fuel substitute, mainly because of viscosity problems. Additional problems are the reliability of product quality in bulk quantities and filter plugging at low temperatures due to crystallization. Therefore, plant oils must be transesterified with short-chain alcohols like methanol or ethanol to yield the FAME and FAEE constituents of biodiesel. This transesterification process and the subsequent purification steps are cost intensive and energy consuming, thereby reducing the possible energy yield and increasing the price. (3) FAMES and FAEEs have comparable chemical and physical fuel properties and engine performances (Peterson *et al.*, 1995), but for economic reasons, only FAMES are currently produced on an industrial scale due to the much lower price of methanol compared to ethanol. Methanol, however, is currently mainly produced from natural gas. Thus, FAME-based biodiesel is not a truly renewable product since the alcohol component is of fossil origin. Furthermore, methanol is highly toxic and hazardous, and its use requires special precautions. Use of bioethanol for production of FAEE-based biodiesel would result in a fully sustainable fuel, but only at the expense of much higher production costs. (4) The major limitation impeding a more widespread use of biodiesel is the extensive acreage needed for production of oilseed crops. The yield of biodiesel from rapeseed is only 1300 l ha<sup>-1</sup>, since only the seed oil is used for biodiesel production, whereas the other, major part of the plant biomass is not used for this purpose. Furthermore, oilseed crops like rapeseed and soybean are not self-compatible; therefore, their cultivation requires a frequent crop-rotation regime. In consequence, biodiesel based on oilseed crops will probably not be able to substitute more than 5–15 % of petroleum-based diesel in the future.

A recent study assessing the use of bioethanol for fuel came to the conclusion that large-scale use will require a cellulose-based technology (Farrell *et al.*, 2006). A substantial increase of biodiesel production and a more significant substitution of petroleum-based diesel fuel in the future will probably only be feasible when processes are developed enabling biodiesel synthesis from bulk plant materials such as sugars and starch, and in particular cellulose and hemicellulose.

Intracytoplasmic storage lipid accumulation in the Gram-negative bacterium *Acinetobacter baylyi* strain ADP1 (formerly *Acinetobacter* sp. strain ADP1; Vaneechoutte *et al.*, 2006) is mediated by the wax ester synthase/acyl-coenzyme A: diacylglycerol acyltransferase (WS/DGAT; the *atfA* gene product). This unspecific acyltransferase simultaneously

synthesizes wax esters and TAGs by utilizing long-chain fatty alcohols or diacylglycerols and fatty acid coenzyme A thioesters (acyl-CoA) as substrates (Kalscheuer & Steinbüchel, 2003). Biochemical characterization of WS/DGAT revealed that this acyltransferase exhibits an extremely low acyl acceptor molecule specificity *in vitro*. The remarkably broad substrate range of WS/DGAT comprises short chain-length up to very long chain-length linear primary alkyl alcohols; cyclic, phenolic and secondary alkyl alcohols; diols and dithiols; mono- and diacylglycerols as well as sterols (Kalscheuer *et al.*, 2003, 2004; Stöveken *et al.*, 2005; Uthoff *et al.*, 2005). By expression of WS/DGAT in different recombinant hosts, this substrate promiscuity has already been exploited to synthesize various fatty acid ester molecules *in vivo*. The type of fatty acid ester synthesized by WS/DGAT was determined by the physiological background of the expression host regarding the provision of substrates accomplished by natural metabolism, medium supplementation or genetic engineering. Examples of those recombinantly synthesized fatty acid ester derivatives are wax esters in recombinant *Pseudomonas citronellolis* (Kalscheuer & Steinbüchel, 2003), wax esters and fatty acid butyl esters (FABEs) in recombinant *Escherichia coli* (Kalscheuer *et al.*, 2006), wax diesters and wax thioesters in the mutant *A. baylyi* strain ADP1*acr1*ΔKm (Kalscheuer *et al.*, 2003; Uthoff *et al.*, 2005), and TAGs, FAEEs and fatty acid isoamyl esters (FAIEs) in recombinant *Saccharomyces cerevisiae* (Kalscheuer *et al.*, 2004). Although only trace amounts were produced, recombinant biosynthesis of FAEEs and FAIEs in yeast as well as FABEs in *E. coli* indicated that production of biodiesel-appropriate fatty acid monoalkyl esters might in principle be feasible by using recombinant WS/DGAT-expressing micro-organisms. The objective of our present study was thus the development of a microbial process for the production of FAEEs for use as biodiesel from simple and renewable carbon sources. For this approach, the natural WS/DGAT host *A. baylyi* strain ADP1 was not a suitable candidate since it is a strictly aerobic bacterium not able to form ethanol. We therefore established FAEE biosynthesis in recombinant *E. coli* by coexpression of the ethanol production genes from the ethanol-producing fermentative bacterium *Zymomonas mobilis* in combination with the WS/DGAT gene from *A. baylyi* strain ADP1.

## METHODS

**Strains, plasmids and cultivation conditions.** *Escherichia coli* TOP10 (Invitrogen) was used in this study. The plasmids used are pLOI297 harbouring the *Zymomonas mobilis* genes for pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adhB*) cloned in pUC18 collinear to the *lacZ* promoter (Alterthum & Ingram, 1989), and pKS::atfA and pBBR1MCS-2::atfA harbouring the WS/DGAT gene from *A. baylyi* strain ADP1 collinear to the *lacZ* promoter in pBluescript KS<sup>-</sup> or pBBR1MCS-2, respectively (Kalscheuer & Steinbüchel, 2003). The construction of plasmid pMicrodiesel is described in Results.

Recombinant strains of *E. coli* were cultivated in LB medium (0.5 %, w/v, yeast extract, 1 %, w/v, tryptone and 1 %, w/v, NaCl) containing 1 mM IPTG and 2 % (w/v) glucose at 37 °C in the presence of ampicillin

(75 mg l<sup>-1</sup>) and kanamycin (50 mg l<sup>-1</sup>) for selection of pLOI297, pKS::*atfA* and pMicrodiesel or pBBR1MCS-2::*atfA*, respectively. Where indicated, sodium oleate was added from a 10% (w/v) stock solution in H<sub>2</sub>O to a final concentration of 0.1 or 0.2% (w/v). Cells were grown aerobically in 300 ml baffled Erlenmeyer flasks containing 50 ml medium on an orbital shaker (130 r.p.m.).

**Bioreactor cultivation.** Fermentation experiments were done in a 2 litre stirred bioreactor (B. Braun Biotech International) with an initial volume of 1.5 l LB medium containing 0.2% (w/v) sodium oleate, 2% (w/v) glucose, 1 mM IPTG and appropriate antibiotics for plasmid selection (see above). Cultivations were done at 37 °C and at a stirring rate of 200 r.p.m. If not stated otherwise, the pH was controlled at 7.0 by automated addition of 4 M HCl or NaOH. Cells were cultivated either aerobically (aeration rate 3 vvm), under restricted oxygen conditions (aeration rate 0.75 vvm), or anaerobically. Inoculum was 5% (v/v) of saturated overnight cultures.

**Thin-layer chromatography.** TLC analysis of lipid extracts from whole cells was done as described previously (Kalscheuer & Steinbüchel, 2003) using the solvent system hexane/diethyl ether/acetic acid (90:7.5:1, by vol.). Lipids were visualized by spraying with 40% (v/v) sulfuric acid and charring. Ethyl oleate was purchased from Sigma-Aldrich Chemie and used as reference substance for FAEEs.

**GC and GC/MS analysis of FAEEs.** For quantification of FAEEs, 5 ml culture broth was extracted with 5 ml chloroform/methanol (2:1, v/v) by vigorous vortexing for 5 min. After phase separation, the organic phase was withdrawn, evaporated to dryness, and redissolved in 1 ml chloroform/methanol (2:1, v/v). FAEEs were analysed by GC on an Agilent 6850 GC (Agilent Technologies) equipped with a BP21 capillary column (50 m × 0.22 mm, film thickness 250 nm; SGE) and a flame-ionization detector (Agilent Technologies). A 2 µl portion of the organic phase was analysed after split injection (1:20); hydrogen (constant flow 0.6 ml min<sup>-1</sup>) was used as carrier gas. The temperatures of the injector and detector were 250 and 275 °C, respectively. The following temperature programme was applied: 120 °C for 5 min, increase of 3 °C min<sup>-1</sup> to 180 °C, increase of 10 °C min<sup>-1</sup> to 220 °C, 220 °C for 31 min. Identification and quantification were done by using authentic FAEE standards.

For coupled GC/MS analysis, FAEEs were purified by preparative TLC. GC/MS analysis of FAEEs dissolved in chloroform was done on a Series 6890 GC system equipped with a Series 5973 EI MSD mass-selective detector (Hewlett Packard). A 3 µl portion of the organic phase was analysed after splitless injection on a BP21 capillary column (50 m × 0.22 mm, film thickness 250 nm; SGE). Helium (constant flow 0.6 ml min<sup>-1</sup>) was used as carrier gas. The temperatures of the injector and detector were 250 °C and 240 °C, respectively. The same temperature programme as described for GC analysis was applied. Data were evaluated by using the NIST-Mass Spectral Search Program (Stein *et al.*, 1998).

**Ethanol quantification.** Ethanol in cell-free aqueous culture supernatants was determined by GC essentially as described above for FAEE quantification, but applying a modified temperature programme: 70 °C for 20 min, increase of 10 °C min<sup>-1</sup> to 180 °C, increase of 10 °C min<sup>-1</sup> to 220 °C, 220 °C for 25 min.

**General molecular biological techniques.** Standard molecular biological techniques were applied according to Sambrook *et al.* (1989).

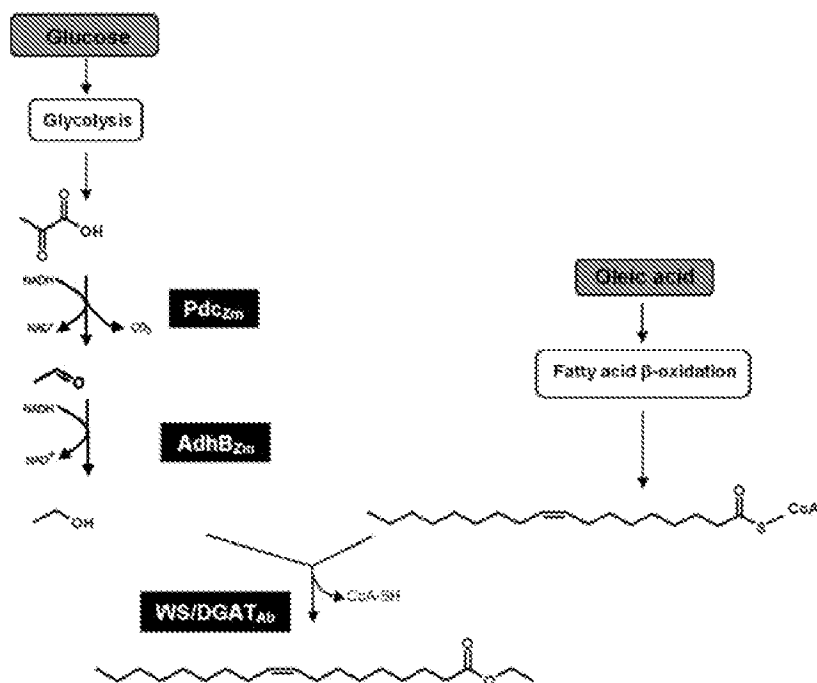
## RESULTS

### Establishment of FAEE biosynthesis in recombinant *E. coli* TOP10 by metabolic engineering

The unspecific acyltransferase WS/DGAT from *A. baylyi* strain ADP1 has been shown to be capable of utilizing ethanol to some extent as an acyl acceptor substrate (Kalscheuer *et al.*, 2004; Stöveken *et al.*, 2005). However, heterologous expression of the WS/DGAT-encoding *atfA* gene alone from pBBR1MCS-2::*atfA* did not result in FAEE formation in *E. coli* TOP10 during cultivation in LB medium containing 2% (w/v) glucose, 1 mM IPTG and 0.1% (w/v) sodium oleate under either aerobic or anaerobic conditions (data not shown). Although *E. coli* is known to form ethanol during mixed acid fermentation, obviously ethanol synthesis and/or uptake of oleic acid from the medium and activation to the acyl-CoA thioester were too inefficient to support detectable FAEE formation under anaerobic conditions. However, increased ethanol production has been achieved in *E. coli* upon heterologous expression of pyruvate decarboxylase (the *pdc* gene product) and alcohol dehydrogenase (the *adhB* gene product) from the strictly anaerobic ethanologenic Gram-negative bacterium *Zymomonas mobilis*. Using this system, efficient ethanol biosynthesis was achieved from glucose via the glycolysis product pyruvate even under aerobic conditions (Ingram *et al.*, 1987; Alterthum & Ingram, 1989).

We therefore attempted to establish FAEE biosynthesis in a recombinant *E. coli* by combining expression of the *Z. mobilis* genes *pdc* and *adhB* and of the *atfA* gene from *A. baylyi* strain ADP (Fig. 1) using plasmids pLOI297 (*pdc* and *adhB*) and pBBR1MCS-2::*atfA*. Recombinant strains carrying either plasmid alone did not exhibit FAEE levels detectable by TLC (Fig. 2a, lanes 1 and 2). However, coexpression of all three relevant genes in a strain carrying both plasmids resulted in significant FAEE formation (Fig. 2a, lane 3). FAEE biosynthesis was strictly dependent on the presence of sodium oleate in the medium (data not shown). Growth of strains harbouring plasmid pLOI297 was very poor in LB medium without glucose addition, and FAEE synthesis was not observable in *E. coli* TOP10 harbouring both plasmids under these conditions (data not shown). The FAEEs formed were accumulated intracellularly, and no significant extracellular lipids were found in cell-free culture supernatants (data not shown).

GC/MS analysis of FAEE isolated from *E. coli* TOP10(pBBR1MCS-2::*atfA*+pLOI297) cultivated in medium supplemented with sodium oleate revealed a mixture of esters mainly consisting of ethyl oleate plus minor amounts of ethyl palmitate and ethyl palmitoleate (Fig. 2b). The presence of ethyl palmitate indicated that also some fatty acids derived from *de novo* fatty acid biosynthesis were channelled into FAEE production. When technical-grade sodium oleate (content ~80%) was used for cultivations at a larger scale, low amounts of ethyl



**Fig. 1.** Pathway of FAEE biosynthesis in recombinant *E. coli*. FAEE formation was achieved by coexpression of the ethanolic enzymes pyruvate decarboxylase (Pdc) and alcohol dehydrogenase (AdhB) from *Z. mobilis* and the unspecific acyltransferase WS/DGAT from *A. baylyi* strain ADP1.

myristate (C14:0-ethyl ester,  $m/z = 256$  [ $C_{16}H_{32}O_2$ ] $^+$ ), ethyl myristoleate (C14:1-ethyl ester,  $m/z = 254$  [ $C_{16}H_{30}O_2$ ] $^+$ ) and ethyl linoleate (C18:2-ethyl ester,  $m/z = 308$  [ $C_{20}H_{36}O_2$ ] $^+$ ) were also observed due to the presence of the corresponding fatty acid impurities (data not shown).

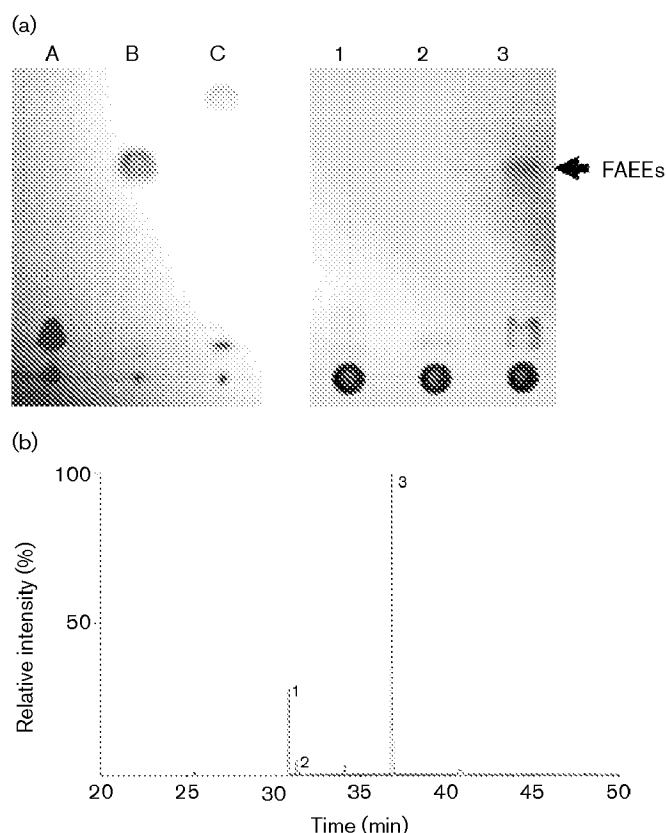
#### Batch fermentations of *E. coli* TOP10(pBBR1MCS-2::atfA+pLOI297) for FAEE production

The shake-flask experiments under aerobic conditions described above clearly proved the concept that FAEE biosynthesis is feasible in recombinant *E. coli*. Oxygen availability might have a great influence on the ethanol synthesis rate in this recombinant system, with low-oxygen conditions supposed to favour ethanol formation, and thus might also have a profound impact on the FAEE biosynthesis rate. We therefore cultivated *E. coli* TOP10(pBBR1MCS-2::atfA+pLOI297) under conditions permissive for FAEE formation with different controlled oxygen conditions (Fig. 3). Although ethanol production was slightly higher under anaerobic conditions (maximal  $4.39 \text{ g l}^{-1}$  after 17 h), only a very low FAEE content was observed, plateauing already after 18 h at a concentration of  $0.05\text{--}0.07 \text{ g l}^{-1}$  (Fig. 3b). In contrast, FAEE biosynthesis was significantly higher under aerobic conditions (aeration rate 3 vvm). FAEE formation was not restricted to a certain growth phase but continued throughout the cultivation period, finally reaching  $0.26 \text{ g l}^{-1}$  after 48 h (Fig. 3a). With a final cellular dry biomass of  $4.3 \text{ g l}^{-1}$  obtained by aerobic cultivation this corresponds to a cellular FAEE content of 6.1 % (w/w). When the cells were cultivated under oxygen-restricted conditions (aeration rate  $0.75 \text{ vvm}$ ) a final FAEE

concentration of  $0.16 \text{ g l}^{-1}$  was obtained after 48 h (data not shown). Under all three cultivation conditions ethanol concentration reached a maximum after 15–20 h cultivation, after which a rapid decrease was unexpectedly observed (Fig. 3a, b), which has not to our knowledge been described before for ethanologenic *E. coli* strains employing the *Z. mobilis* *pdc* and *adhB* genes for recombinant ethanol synthesis.

#### Construction of plasmid pMicrodiesel

To simplify the process by reducing the number of antibiotics required for plasmid stabilization and to potentially increase FAEE yield by providing all three relevant genes on a high-copy-number vector, plasmid pMicrodiesel was constructed. For this, a 3.2 kbp DNA fragment was amplified from plasmid pLOI297 by tailored PCR using the oligonucleotides 5'-AAAGGATCCGCGCA-ACGTAATTAATGTGAGTT-3' (forward primer) and 5'-TTTGGATCCCCAAATGGCAAATTATT-3' (reverse primer) introducing *Bam*HI restriction sites (underlined). This 3.2 kbp *Bam*HI fragment, which comprised the *Z. mobilis* genes *pdc* and *adhB* and the upstream *lacZ* promoter region, was cloned into *Bam*HI-linearized pKS::atfA, a derivative of the high-copy-number plasmid pBluescript KS $^-$  (Kalscheuer & Steinbüchel, 2003), yielding pMicrodiesel (Fig. 4). The orientation of *atfA*, *pdc* and *adhB* was determined by *Eco*RI restriction and DNA sequence analysis. Plasmid pMicrodiesel carried all three genes relevant for FAEE synthesis in a collinear orientation, with *atfA* driven by a *lacZ* promoter and with *pdc* and *adhB* controlled by a second *lacZ* promoter, thereby ensuring effective transcription of all three genes.



**Fig. 2.** Chemical analysis of FAEEs produced by recombinant *E. coli* TOP10. (a) TLC analysis of intracellular lipids accumulated by recombinant *E. coli* TOP10. Cells were cultivated aerobically in shake flasks for 24 h at 37 °C in LB medium containing 2% (w/v) glucose, 0.1% (w/v) sodium oleate, 1 mM IPTG and appropriate antibiotics as described in Methods. A, oleic acid; B, ethyl oleate; C, oleyl oleate; 1, *E. coli* TOP10(pLOI297); 2, *E. coli* TOP10(pBBR1MCS-2::atfA); 3, *E. coli* TOP10(pBBR1MCS-2::atfA+pLOI297). Total lipid extracts each obtained from 1.5 mg lyophilized cells were applied in lanes 1–3. (b) Total ion profile of GC/MS analysis of FAEEs isolated from *E. coli* TOP10(pBBR1MCS-2::atfA+pLOI297). Cells were cultivated as described above. FAEEs were purified by preparative TLC. Identified substances: 1, ethyl palmitate (C16:0-ethyl ester,  $m/z=284$  [ $C_{18}H_{36}O_2$ ] $^+$ ); 2, ethyl palmitoleate (C16:1-ethyl ester,  $m/z=282$  [ $C_{18}H_{34}O_2$ ] $^+$ ); 3, ethyl oleate (C18:1-ethyl ester,  $m/z=310$  [ $C_{20}H_{38}O_2$ ] $^+$ ).

### Fed-batch fermentation of *E. coli* TOP10(pMicrodiesel) for FAEE production

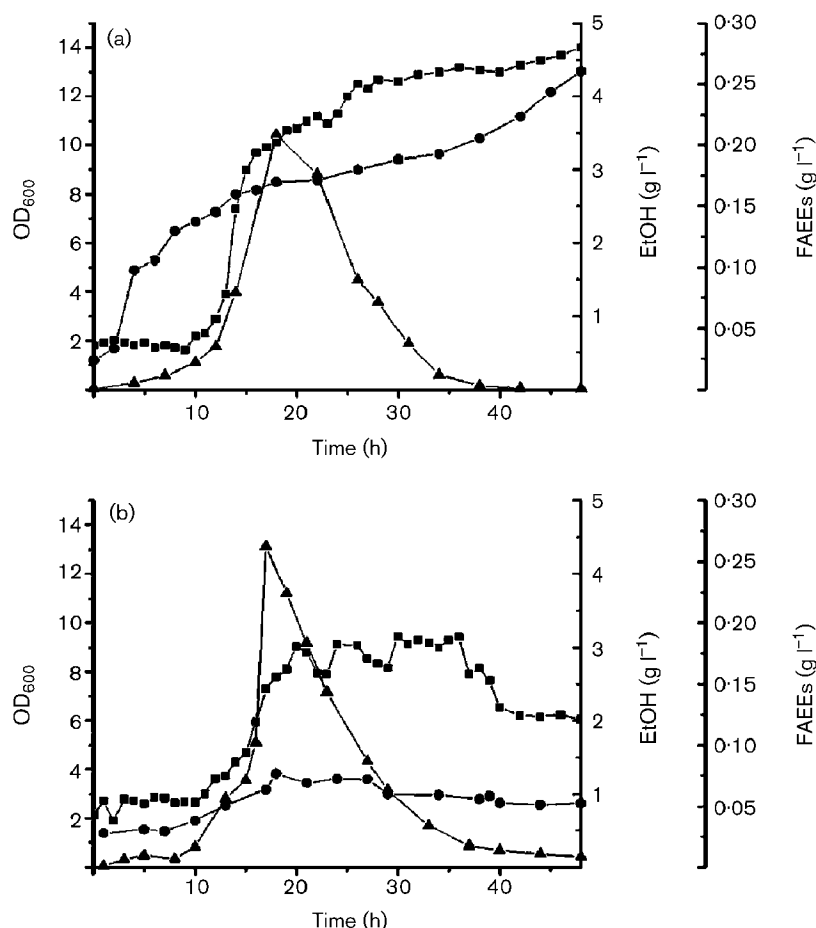
Shake-flask experiments with *E. coli* TOP10 harbouring either pMicrodiesel alone or pLOI297 plus pBBR1MCS-2::atfA revealed a more than twofold higher FAEE production using the newly constructed plasmid pMicrodiesel ( $0.64 \text{ g l}^{-1}$  compared to  $0.26 \text{ g l}^{-1}$ ) whereas ethanol concentrations were similar. This indicated the positive influence of provision of all three relevant genes on a high copy-number vector and, as consequence, potentially higher expression rates on FAEE yield.

We then aspired to further optimize FAEE production by *E. coli* TOP10(pMicrodiesel), employing an aerobic fed-batch fermentation regime. Initial optimization experiments revealed that no regulation of medium pH during cultivation, resulting in a slightly acidic pH of 6.0–6.5 at the end, rather than a strict regulation at pH 7.0, might be favourable for FAEE biosynthesis (data not shown). Thus, the pH value was only roughly regulated automatically between 6.0 and 8.5 during the following fed-batch fermentation experiment (Fig. 5). To avoid carbon limitation, glucose was fed several times during the cultivation period. FAEE concentration continuously increased throughout the fermentation process, whereas its composition remained relatively constant (similar to the results shown in Fig. 2b). Employing this fed-batch strategy, a final FAEE content of  $1.28 \text{ g l}^{-1}$  was achieved after 72 h, which was about five times higher compared to aerobic batch fermentation of the *E. coli* TOP10 strain harbouring pLOI297 plus pBBR1MCS-2::atfA (Fig. 3a). With a final cellular dry biomass of  $4.9 \text{ g l}^{-1}$  this corresponds to an impressive cellular FAEE content of 26% (w/w). Referred to the initial amount of  $2 \text{ g l}^{-1}$  present in the medium at the beginning of the cultivation, sodium oleate was converted to FAEEs with an efficiency of 62.7% on a molar basis.

## DISCUSSION

Biodiesel is an interesting alternative energy source and is used as substitute for petroleum-based diesel. Offering numerous environmental benefits, it has attracted broad public interest and is being produced in increasing amounts (see Introduction). However, a broader use of biodiesel and a more significant substitution of petroleum-based fuels in the future will only be possible if production processes are developed that are not solely based on oilseed crops but on more bulk plant materials like cellulose. Toward this goal, we report here on a novel approach to establish biotechnological production of biodiesel using metabolically engineered micro-organisms, which we refer to as Microdiesel. The early optimization studies described here revealed FAEE yields of up to 26% of the bacterial dry biomass. Although these yields are still far below the needs for an industrial process, this study has clearly proved the feasibility, in principle, of this novel approach. Therefore, the present study might open new avenues potentially enabling microbial production of fuel equivalents from cheap and readily available renewable bulk plant materials like sugars, starch, cellulose or hemicellulose in the future.

Microbial FAEE biosynthesis for Microdiesel production is based on the exploitation of the extraordinarily low substrate specificity of the acyltransferase (WS/DGAT) of *A. baylyi* strain ADP1, which in its natural host mediates wax ester and TAG biosynthesis from acyl-CoA thioesters plus long chain-length fatty alcohols or diacylglycerols (Kalscheuer & Steinbüchel, 2003). *E. coli* does not produce such substances by its natural metabolism; however, recombinant strains enabled to produce large amounts of



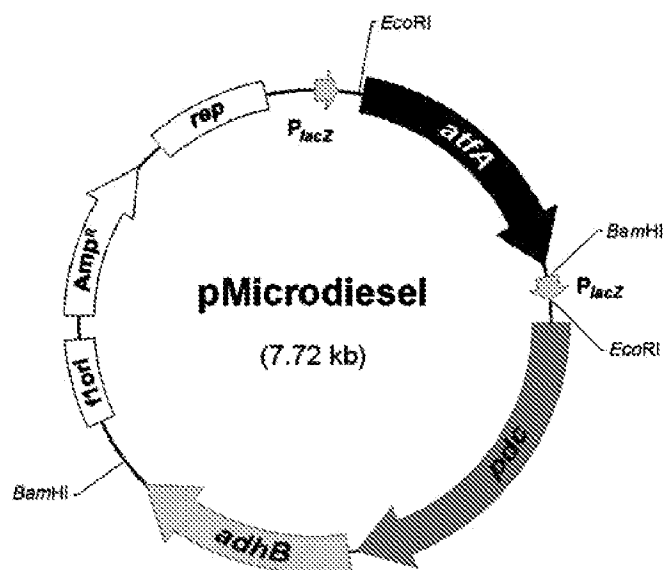
**Fig. 3.** FAEE production during batch fermentations of *E. coli* TOP10(pBBR1MCS-2::attA+pLOI297). Cultivations were done in a 2 litre stirred bioreactor initially filled with 1.5 l LB medium containing 0.2% (w/v) sodium oleate, 2% (w/v) glucose, 1 mM IPTG, 75 mg ampicillin l<sup>-1</sup> and 50 mg kanamycin l<sup>-1</sup> as described in Methods. Sodium oleate causes turbidity of the medium, explaining the high initial optical densities. ■, OD<sub>600</sub>; ▲, ethanol concentration; ●, FAEE concentration. (a) Cultivation under aerobic conditions (aeration rate 3 vvm). (b) Cultivation under anaerobic conditions.

ethanol and simultaneously expressing WS/DGAT provided an unusual, alternative substrate for this acyltransferase. This resulted in production of substantial amounts of FAEEs utilizing WS/DGAT's substrate promiscuity.

*E. coli* forms ethanol, among other fermentation products, during mixed acid fermentation under anaerobic conditions from acetyl-CoA via two sequential NADH-dependent reductions catalysed by a multifunctional alcohol dehydrogenase (the *adhE* gene product) (Goodlove *et al.*, 1989; Kessler *et al.*, 1992). However, ethanol levels naturally occurring in *E. coli* under anaerobic conditions are probably not sufficient to support formation of significant amounts of FAEE. In addition, several other fermentation products besides ethanol occur in substantial amounts. By using a recombinant system employing *Z. mobilis* pyruvate decarboxylase and alcohol dehydrogenase, this limitation was circumvented, resulting in substantial amounts of ethanol under aerobic conditions, which is in accordance with previous reports (Ingram *et al.*, 1987; Alterthum & Ingram, 1989). In fed-batch fermentations conducted under controlled aeration rates, the highest FAEE levels were observed in recombinant *E. coli* under aerobic conditions (approximately five times higher compared to anaerobic conditions) although ethanol levels were similar. This indicates that

uptake of exogenous fatty acids from the medium and their activation to the corresponding acyl-CoA thioesters is probably another factor limiting Microdiesel production in *E. coli* under anaerobic conditions.

Although an impressive FAEE content as high as 26% of the cellular dry weight was finally obtained, *E. coli* is not ideal for Microdiesel production for various reasons. Although the occurrence of ethyl palmitate as a minor constituent indicated that fatty acids derived from *de novo* fatty acid biosynthesis were channelled into FAEE production, substantial FAEE biosynthesis was strictly dependent on supplementation of exogenous fatty acids. This indicates that *de novo* fatty acid biosynthesis, in contrast to fatty acid  $\beta$ -oxidation, can not provide sufficient intracellular acyl substrates for WS/DGAT-mediated FAEE synthesis. Therefore, it will be challenging to establish Microdiesel production solely from simple bulk plant materials like sugars, cellulose or hemicellulose in the future using *E. coli* as a production platform. As an alternative, storage-lipid-accumulating bacteria, in particular those of the actinomycete group, may be used; these bacteria are capable of synthesizing from simple carbon sources like glucose under growth-restricted conditions remarkably high amounts of fatty acids (up to ~70% of the cellular dry weight) and



**Fig. 4.** Map of plasmid pMicrodiesel. Relevant characteristics: *rep*, origin of replication; Amp<sup>R</sup>, ampicillin-resistance gene; P<sub>lacZ</sub>, *lacZ* promoter; *pdc*, pyruvate decarboxylase gene from *Z. mobilis*; *adhB*, alcohol dehydrogenase gene from *Z. mobilis*; *attA*, WS/DGAT gene from *A. baylyi* strain ADP1.

accumulate them intracellularly as TAGs (Alvarez & Steinbüchel, 2002). If the flux of fatty acids could be directed from TAG towards FAEE biosynthesis by genetic manipulation, storage-lipid-accumulating bacteria might be promising candidates for more simplified Microdiesel production processes in the future. Establishment of recombinant ethanol biosynthesis in these aerobic, non-fermentative bacteria would be a prerequisite for this purpose. In this regard, a recently developed heterologous ethanol production system for Gram-positive bacteria could become of great value and utility (Talarico *et al.*, 2005). Future optimization of biotechnological Microdiesel

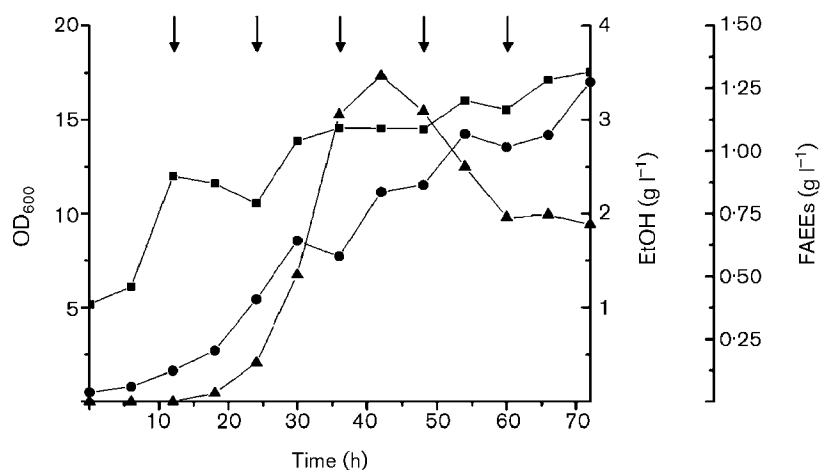
production will also benefit from the progress made in recent years in lignocellulose utilization as feedstock for bioethanol production by recombinant micro-organisms (Dien *et al.*, 2003; Zaldivar *et al.*, 2001).

A further bottleneck in the path towards optimized FAEE levels is the relatively low reaction rate of WS/DGAT with ethanol in comparison with longer chain-length fatty alcohols (C<sub>10</sub>–C<sub>18</sub>) (Kalscheuer *et al.*, 2004; Stöveken *et al.*, 2005). Numerous genes encoding WS/DGAT homologues have been identified in several other bacteria (Kalscheuer & Steinbüchel, 2003). One of those acyltransferases might be more suitable for FAEE production since it may exhibit a higher specificity for ethanol. Alternatively, increase of the reaction rate of WS/DGATs with ethanol may be achieved by enzyme engineering.

Optimized Microdiesel production by engineered micro-organisms could finally offer some major advantages over established conventional production processes. Biotechnological Microdiesel production could be significantly less expensive than conventional biodiesel production if plant products like starch or lignocellulose are used for its production. These plant polymers are not only much cheaper than plant oils, but are also much more abundant, and Microdiesel production will not be restricted to oilseed-producing regions of the world. In contrast to conventional FAME-based biodiesel, Microdiesel is a fully sustainable biofuel completely derived from renewable materials, also avoiding the use of highly toxic methanol. In conclusion, this study provides a basis to achieve more competitive production costs, and therefore a more substantial substitution of petroleum-derived fuels by biofuels in the future.

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**Fig. 5.** FAEE production during fed-batch fermentation of *E. coli* TOP10(pMicrodiesel). Cultivation was done in a 2 litre stirred bioreactor initially filled with 1.5 l LB medium containing 0.2% (w/v) sodium oleate, 2% (w/v) glucose, 1 mM IPTG and 75 mg ampicillin l<sup>-1</sup> under aerobic conditions (aeration rate 3 vvm) as described in Methods. The pH was kept between 6.0 and 8.5 by automated addition of 4 M HCl or NaOH. To prevent carbon limitation, 1 g glucose l<sup>-1</sup> was fed several times during cultivation (indicated by arrows). Sodium oleate causes turbidity of the medium, explaining the high initial optical density. ■, OD<sub>600</sub>; ▲, ethanol concentration; ●, FAEE concentration.

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(54) Title: PRODUCTION OF FATTY ACIDS AND DERIVATIVES THEREOF

(57) Abstract: Genetically engineered microorganisms are provided that produce products from the fatty acid biosynthetic pathway (fatty acid derivatives), as well as methods of their use.



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## **PRODUCTION OF FATTY ACIDS AND DERIVATIVES THEREOF**

### **Cross-reference to Related Applications**

This application claims the benefit of US Provisional Application No. 60/802,016 filed May 19, 2006, US Provisional Application No. 60/801,995 filed May 19, 2006, US Provisional Application 60/908,547 filed March 28, 2007 and PCT application number PCT/US2007/003736 filed February 13, 2007, all of which are herein incorporated by reference.

### **Field**

Genetically engineered microorganisms are provided that produce products from the fatty acid biosynthetic pathway (fatty acid derivatives), as well as methods of their use.

### **Background**

Developments in technology have been accompanied by an increased reliance on fuel sources and such fuel sources are becoming increasingly limited and difficult to acquire. With the burning of fossil fuels taking place at an unprecedented rate, it has likely that the world's fuel demand will soon outweigh the current fuel supplies.

As a result, efforts have been directed toward harnessing sources of renewable energy, such as sunlight, water, wind, and biomass. The use of biomasses to produce new sources of fuel which are not derived from petroleum sources, (i.e. biofuel) has emerged as one alternative option. Biofuel (biodiesel) is a biodegradable, clean-burning combustible fuel made of long chain alkanes and esters. Biodiesel can be used in most internal combustion diesel engines in either a pure form, which is referred to as "neat" biodiesel, or as a mix in any concentration with regular petroleum diesel. Current methods of making biodiesel involve transesterification of triacylglycerides (mainly vegetable oil) which leads to a mixture of fatty acid esters and the unwanted side product glycerin, thus, providing a product that is heterogeneous and a waste product that causes economic inefficiencies.

### Summary

Disclosed herein are recombinant microorganisms that are capable of synthesizing products derived from the fatty acid biosynthetic pathway (fatty acid derivatives), and optionally releasing such products into the fermentation broth. Such fatty acid derivatives are useful, *inter alia*, as biofuels and specialty chemicals. These biofuels and specialty chemicals can be used to make additional products, such as nutritional supplements, polymers, paraffin replacements, and personal care products.

The recombinant microorganisms disclosed herein can be engineered to yield various fatty acid derivatives including, but not limited to, short chain alcohols such as ethanol, propanol isopropanol and butanol, fatty alcohols, fatty acid esters, hydrocarbons and wax esters.

In one example, the disclosure provides a method for modifying a microorganism so that it produces, and optionally releases, fatty acid derivatives generated from a renewable carbon source. Such microorganisms are genetically engineered, for example, by introducing an exogenous DNA sequence encoding one or more proteins capable of metabolizing a renewable carbon source to produce, and in some examples secrete, a fatty acid derivative. The modified microorganisms can then be used in a fermentation process to produce useful fatty acid derivatives using the renewable carbon source (biomass) as a starting material. In some examples, an existing genetically tractable microorganism is used because of the ease of engineering its pathways for controlling growth, production and reducing or eliminating side reactions that reduce biosynthetic pathway efficiencies. In addition, such modified microorganisms can be used to consume renewable carbon sources in order to generate fuels that can be directly used as biofuels, without the need for special methods for storage, or transportation. In other examples, microorganisms that naturally produce hydrocarbons are engineered to overproduce hydrocarbons by expressing exogenous nucleic acid sequences that increase fatty acid production.

Provided herein are microorganisms that produce fatty acid derivatives having defined carbon chain length, branching, and saturation levels. In particular examples, the production of homogeneous products decreases the overall cost associated with fermentation and separation. In some examples microorganisms

including one or more exogenous nucleic acid sequences encoding at least one thioesterase (EC 3.1.2.14), and at least one wax synthase (EC 2.3.1.75) are provided. In other examples microorganisms are provided that include one or more exogenous nucleic acid sequences encoding at least one thioesterase (EC 3.1.2.14) and at least one alcohol acetyltransferase (2.3.1.84). In yet other examples, microorganisms including one or more exogenous nucleic acid sequences encoding at least one thioesterase (EC 3.1.2.14), at least one acyl-CoA reductase (EC 1.2.1.50) and at least one alcohol dehydrogenase (EC 1.1.1.1) are provided. Microorganisms expressing one or more exogenous nucleic acid sequences encoding at least one thioesterase (EC 3.1.2.14) and at least one fatty alcohol forming acyl-CoA reductase (1.1.1.\*) are also provided. The thioesterase peptides encoded by the exogenous nucleic acid sequences can be chosen to provide homogeneous products.

In some examples the microorganism that is engineered to produce the fatty acid derivative is *E. coli*, *Z. mobilis*, *Rhodococcus opacus*, *Ralstonia eutropha*, *Vibrio furnissii*, *Saccharomyces cerevisiae*, *Lactococcus lactis*, *Streptomyces*, *Stenotrophomonas maltophilia*, *Pseudomonas* or *Micrococcus leuteus* and their relatives.

In other examples microorganisms that produce hydrocarbons endogenously can be engineered to overproduce hydrocarbons by optimizing the fatty acid biosynthetic pathway as described herein. Exemplary microorganisms that are known to produce hydrocarbons and can be engineered to over-produce hydrocarbons using the teachings provided herein include *Arthrobacter sp.*, *Bacillus sp.*, *Botryococcus braunii*, *Chromatium sp.*, *Cladosporium resina* (ATCC22711), *Clostridium pasteurianum* VKM, *Clostridium tenanomorphum*, *Clostridium acidurici*, *Corynebacterium species*, *cyanobacterial species* (*Nostoc muscorum*, *Anacystis* (*Synechococcus*) *nidulans*, *Phormidium luridum*, *Chlorogloea fritschii*, *Trichodesmium erythaeum*, *Oscillatoria williamsii*, *Microcoleus chthonoplaseis*, *Coccochloris elabens*, *Agmenellum quadruplicatum*, *Plectonema terebrans*, *M. vaginatus*, and *C. scopulorum*), *Desulfovibrio desulfuricans* (ATCC29577), *Kineococcus radiotolerans* (BAA-149), *Micrococcus luteus* (FD533, ATCC 272, 381, 382, ISU, 540, 4698, 7468, 27141), *Micrococcus sp.* (ATCC 146, 398, 401, 533), *Micrococcus roseus* (ATCC 412, 416, 516), *Micrococcus lysodeikticus*,

*Mycobacterium species*, *Penicillium sp.*, *Aspergillus sp.*, *Trichoderma virida*, *Pullularia pullulans*, *Jeotgalicoccus sp.* (*M. candidans*) (ATCC 8456), *Rhodopseudomonas spheroids* *Chlorobium sp.*, *Rhodospirillum rubrum* (ATCC11170), *Rhodomicrobium vannielii*, *Stenotrophomonas maltophilia* (ATCC 13637, 17444, 17445, 17666, 17668, 17673, 17674, 17679, 17677), *Saccharomycodes ludwigii* (ATCC 22711), *Saccharomyces sp.* (*oviformus*, *ludwigii*, *tropicalis*), *Vibrio furnissii* M1, *Vibrio marinus* MP-1, *Vibrio ponticus*, *Serratia marinarubra*, *Ustilago maydis*, *Ustilago nuda*, *Urocystis agropyri*, *Sphacelotheca reiliana*, and *Tilletia sp.* (*foetida*, *caries*, *controversa*).

In addition to being engineered to express exogenous nucleic acid sequences that allow for the production of fatty acid derivatives, the microorganism can additionally have one or more endogenous genes functionally deleted or attenuated. For example, *ackA* (EC 2.7.2.1), *ackB* (EC 2.7.2.1), *adhE* (EC 1.1.1.1, 1.2.1.10), *fabF* (EC 2.3.1.179), *fabR* (accession NP\_418398), *fadE* (EC 1.3.99.3, 1.3.99.-), GST (EC 6.3.2.3), *gpsA* (EC 1.1.1.94), *ldhA* (EC 1.1.1.28), *pflB* (EC 2.3.1.54), *plsB* (EC 2.3.1.15), *poxB* (EC 1.2.2.2), *pta* (EC 2.3.1.8), glutathione synthase (EC 6.3.2.3) and combinations thereof can be attenuated.

In addition to being engineered to express exogenous nucleic acid sequences that allow for the production of fatty acid derivatives, the microorganism can additionally have one or more additional genes over-expressed. For example, *pdh*, *panK*, *aceEF* (encoding the E1p dehydrogenase component and the E2p dihydrolipoamide acyltransferase component of the pyruvate and 2-oxoglutarate dehydrogenase complexes, Accessions: NP\_414656, NP\_414657, EC: 1.2.4.1. 2.3.1.61, 2.3.1.12), *accABCD* /*fabH* /*fabD* /*fabG* /*acpP* /*fabF* (encoding FAS, Accessions: CAD85557, CAD85558, NP\_842277, NP\_841683, NP\_415613, EC: 2.3.1.180, 2.3.1.39, 1.1.1.100, 1.6.5.3, 2.3.1.179), genes encoding fatty-acyl-coA reductases (Accessions: AAC45217, EC 1.2.1.-), *UdhA* or similar genes (encoding pyridine nucleotide transhydrogenase, Accession: CAA46822, EC: 1.6.1.1) and genes encoding fatty-acyl-coA reductases (Accessions: AAC45217, EC 1.2.1.-).

In some examples, the microorganisms described herein produce at least 1 mg of fatty acid derivative per liter fermentation broth. In other examples the microorganisms produce at least 100 mg/L, 500 mg/L, 1 g/L, 5 g/L, 10 g/L, 20 g/L,

25 g/L, 30 g/L, 35 g/L, 40 g/L, 50 g/L, 100 g/L, or 120 g/L of fatty acid derivative per liter fermentation broth. In some examples, the fatty acid derivative is produced and released from the microorganism and in yet other examples the microorganism is lysed prior to separation of the product.

In some examples, the fatty acid derivative includes a carbon chain that is at least 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, or 34 carbons long. In some examples at least 50%, 60%, 70%, 80%, 85%, 90%, or 95% of the fatty acid derivative product made contains a carbon chain that is 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, or 34 carbons long. In yet other examples, at least 60%, 70%, 80%, 85%, 90%, or 95% of the fatty acid derivative product contain 1, 2, 3, 4, or 5, points of unsaturation

Also provided are methods of producing fatty acid derivatives. These methods include culturing the microorganisms described herein and separating the product from the fermentation broth.

These and other examples are described further in the following detailed description.

### **Brief Description of the Figures**

**Fig. 1** shows the FAS biosynthetic pathway.

**Fig. 2** shows biosynthetic pathways that produce waxes. Waxes can be produced in a host cell using alcohols produced within the host cell or they can be produced by adding exogenous alcohols in the medium. A microorganism designed to produce waxes will produce wax synthase enzymes (EC 2.3.1.75) using exogenous nucleic acid sequences as well as thioesterase (EC 3.1.2.14) sequences. Other enzymes that can be also modulated to increase the production of waxes include enzymes involved in fatty acid synthesis (FAS enzymes EC 2.3.1.85), acyl-CoA synthase (EC 2.3.1.86), fatty alcohol forming acyl-CoA reductase (EC 1.1.1.\*), acyl-CoA reductase (1.2.1.50) and alcohol dehydrogenase (EC 1.1.1.1).

**Fig. 3** shows biosynthetic pathways that produce fatty alcohols. Fatty alcohols having defined carbon chain lengths can be produced by expressing exogenous nucleic acid sequences encoding thioesterases (EC 3.1.2.14), and combinations of acyl-CoA reductases (EC 1.2.1.50), alcohol dehydrogenases (EC

1.1.1.1) and fatty alcohol forming acyl-CoA reductases (FAR, EC 1.1.1.\*). Other enzymes that can be also modulated to increase the production of fatty alcohols include enzymes involved in fatty acid synthesis (FAS enzymes EC 2.3.1.85), and acyl-CoA synthase (EC 2.3.1.86).

**Fig. 4** shows biosynthetic pathways that produce fatty acids esters. Fatty acids esters having defined carbon chain lengths can be produced by exogenously expressing various thioesterases (EC 3.1.2.14), combinations of acyl-CoA reductase (1.2.1.50), alcohol dehydrogenases (EC 1.1.1.1), and fatty alcohol forming Acyl-CoA reductase (FAR, EC 1.1.1.\*), as well as, acetyl transferase (EC 2.3.1.84). Other enzymes that can be modulated to increase the production of fatty acid esters include enzymes involved in fatty acid synthesis (FAS enzymes EC 2.3.1.85), and acyl-CoA synthase (EC 2.3.1.86).

**Fig. 5** shows fatty alcohol production by the strain described in Example 4, co-transformed with pCDFDuet-1-fadD-acr1 and plasmids containing various thioesterase genes. The strains were grown aerobically at 25°C in M9 mineral medium with 0.4% glucose in shake flasks. Saturated C10, C12, C14, C16 and C18 fatty alcohol were identified. Small amounts of C16:1 and C18:1 fatty alcohols were also detected in some samples. Fatty alcohols were extracted from cell pellets using ethyl acetate and derivatized with N-trimethylsilyl (TMS) imidazole to increase detection.

**Fig. 6** shows the release of fatty alcohols from the production strain. Approximately 50% of the fatty alcohol produced was released from the cells when they were grown at 37°C.

**Figs. 7A-7D** show GS-MS spectrum of octyl octanoate (C8C8) produced by a production hosts expressing alcohol acetyl transferase (AATs, EC 2.3.1.84) and production hosts expressing wax synthase (EC 2.3.1.75). **Fig. 7A** shows acetyl acetate extract of strain C41(DE3, ΔfadE/pHZ1.43)/pRSET B+pAS004.114B) wherein the pHZ1.43 plasmid expressed ADP1 (wax synthase). **Fig. 7B** shows acetyl acetate extract of strain C41(DE3, ΔfadE/pHZ1.43)/pRSET B+pAS004.114B) wherein the pHZ1.43 plasmid expressed SAAT. **Fig. 7C** shows acetyl acetate extract of strain C41(DE3, ΔfadE/pHZ1.43)/pRSET B+pAS004.114B) wherein the pHZ1.43 plasmid did not contain ADP1 (wax synthase) or SAAT. **Fig. 7D** shows

the mass spectrum and fragmentation pattern of C8C8 produced by C41(DE3,  $\Delta$ fadE/pHZ1.43)/pRSET B+pAS004.114B) wherein the pHZ1.43 plasmid expressed SAAT).

**Fig. 8** shows the distribution of ethyl esters made when the wax synthase from *A. baylyi* ADP1 (WSadp1) was co-expressed with thioesterase gene from *Cuphea hookeriana* in a production host.

**Figs. 9A and 9B** show chromatograms of GC/MS analysis. **Fig. 9A** shows a chromatogram of the ethyl extract of the culture of *E. coli* LS9001 strain transformed with plasmids pCDFDuet-1-fadD-WSadp1, pETDuet-1-tesA. Ethanol was fed to fermentations. **Fig. 9B** shows a chromatogram of ethyl hexadecanoate and ethyl oleate used as reference.

**Fig. 10** shows a table that identifies various genes that can be over-expressed or attenuated to increase fatty acid derivative production. The table also identifies various genes that can be modulated to alter the structure of the fatty acid derivative product. One of ordinary skill in the art will appreciate that some of the genes that are used to alter the structure of the fatty acid derivative will also increase the production of fatty acid derivatives.

### Abbreviations and Terms

The following explanations of terms and methods are provided to better describe the present disclosure and to guide those of ordinary skill in the art in the practice of the present disclosure. As used herein, “comprising” means “including” and the singular forms “a” or “an” or “the” include plural references unless the context clearly dictates otherwise. For example, reference to “comprising a cell” includes one or a plurality of such cells, and reference to “comprising the thioesterase” includes reference to one or more thioesterase peptides and equivalents thereof known to those of ordinary skill in the art, and so forth. The term “or” refers to a single element of stated alternative elements or a combination of two or more elements, unless the context clearly indicates otherwise. For example, the phrase “thioesterase activity or fatty alcohol-forming acyl-CoA reductase activity” refers to thioesterase activity, fatty alcohol forming acyl-CoA reductase activity, or a



combination of both fatty alcohol forming acyl-CoA reductase activity, and thioesterase activity.

Unless explained otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this disclosure belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, suitable methods and materials are described below. The materials, methods, and examples are illustrative only and not intended to be limiting. Other features of the disclosure are apparent from the following detailed description and the claims.

**Accession Numbers:** The accession numbers throughout this description are derived from the NCBI database (National Center for Biotechnology Information) maintained by the National Institute of Health, U.S.A. The accession numbers are as provided in the database on March 27, 2007.

**Enzyme Classification Numbers (EC):** The EC numbers provided throughout this description are derived from the KEGG Ligand database, maintained by the Kyoto Encyclopedia of Genes and Genomics, sponsored in part by the University of Tokyo. The EC numbers are as provided in the database on March 27, 2007.

**Attenuate:** To lessen the impact, activity or strength of something. In one example, the sensitivity of a particular enzyme to feedback inhibition or inhibition caused by a composition that is not a product or a reactant (non-pathway specific feedback) is lessened such that the enzyme activity is not impacted by the presence of a compound. For example, the *fabH* gene and its corresponding amino acid sequence are temperature sensitive and can be altered to decrease the sensitivity to temperature fluctuations. The attenuation of the *fabH* gene can be used when branched amino acids are desired. In another example, an enzyme that has been altered to be less active can be referred to as attenuated.

A functional deletion of an enzyme can be used to attenuate an enzyme. A functional deletion is a mutation, partial or complete deletion, insertion, or other variation made to a gene sequence or a sequence controlling the transcription of a gene sequence, which reduces or inhibits production of the gene product, or renders

the gene product non-functional (*i.e.* the mutation described herein for the *plsB* gene). For example, functional deletion of *fabR* in *E. coli* reduces the repression of the fatty acid biosynthetic pathway and allows *E. coli* to produce more unsaturated fatty acids (UFAs). In some instances a functional deletion is described as a knock-out mutation.

One of ordinary skill in the art will appreciate that there are many methods of attenuating a enzyme activity. For example, attenuation can be accomplished by introducing amino acid sequence changes via altering the nucleic acid sequence, placing the gene under the control of a less active promoter, expressing interfering RNA, ribozymes or antisense sequences that targeting the gene of interest, or through any other technique known in the art.

**Carbon source:** Generally refers to a substrate or compound suitable to be used as a source of carbon for prokaryotic or simple eukaryotic cell growth. Carbon sources can be in various forms, including, but not limited to polymers, carbohydrates, acids, alcohols, aldehydes, ketones, amino acids, peptides, etc. These include, for example, various monosaccharides such as glucose, oligosaccharides, polysaccharides, cellulosic material, xylose, and arabinose, disaccharides, such sucrose, saturated or unsaturated fatty acids, succinate, lactate, acetate, ethanol, etc., or mixtures thereof. The carbon source can additionally be a product of photosynthesis, including, but not limited to glucose.

**cDNA (complementary DNA):** A piece of DNA lacking internal, non-coding segments (introns) and regulatory sequences which determine transcription. cDNA can be synthesized by reverse transcription from messenger RNA extracted from cells.

**Deletion:** The removal of one or more nucleotides from a nucleic acid molecule or one or more amino acids from a protein, the regions on either side being joined together.

**Detectable:** Capable of having an existence or presence ascertained. For example, production of a product from a reactant, for example, the production of C18 fatty acids, is detectable using the method provided in Example 11 below.

**DNA:** Deoxyribonucleic acid. DNA is a long chain polymer which includes the genetic material of most living organisms (some viruses have genes including

ribonucleic acid, RNA). The repeating units in DNA polymers are four different nucleotides, each of which includes one of the four bases, adenine, guanine, cytosine and thymine bound to a deoxyribose sugar to which a phosphate group is attached. Triplets of nucleotides, referred to as codons, in DNA molecules code for amino acid in a peptide. The term codon is also used for the corresponding (and complementary) sequences of three nucleotides in the mRNA into which the DNA sequence is transcribed.

**Endogenous:** As used herein with reference to a nucleic acid molecule and a particular cell or microorganism refers to a nucleic acid sequence or peptide that is in the cell and was not introduced into the cell using recombinant engineering techniques. For example, a gene that was present in the cell when the cell was originally isolated from nature. A gene is still considered endogenous if the control sequences, such as a promoter or enhancer sequences that activate transcription or translation have been altered through recombinant techniques.

**Exogenous:** As used herein with reference to a nucleic acid molecule and a particular cell refers to any nucleic acid molecule that does not originate from that particular cell as found in nature. Thus, a non-naturally-occurring nucleic acid molecule is considered to be exogenous to a cell once introduced into the cell. A nucleic acid molecule that is naturally-occurring also can be exogenous to a particular cell. For example, an entire coding sequence isolated from cell X is an exogenous nucleic acid with respect to cell Y once that coding sequence is introduced into cell Y, even if X and Y are the same cell type.

**Expression:** The process by which a gene's coded information is converted into the structures and functions of a cell, such as a protein, transfer RNA, or ribosomal RNA. Expressed genes include those that are transcribed into mRNA and then translated into protein and those that are transcribed into RNA but not translated into protein (for example, transfer and ribosomal RNAs).

**Fatty ester:** Includes any ester made from a fatty acid. The carbon chains in fatty acids can contain any combination of the modifications described herein. For example, the carbon chain can contain one or more points of unsaturation, one or more points of branching, including cyclic branching, and can be engineered to be short or long. Any alcohol can be used to form fatty acid esters, for example

alcohols derived from the fatty acid biosynthetic pathway, alcohols produced by the production host through non-fatty acid biosynthetic pathways, and alcohols that are supplied in the fermentation broth.

**Fatty acid derivative:** Includes products made in part from the fatty acid biosynthetic pathway of the host organism. The fatty acid biosynthetic pathway includes fatty acid synthase enzymes which can be engineered as described herein to produce fatty acid derivatives, and in some examples can be expressed with additional enzymes to produce fatty acid derivatives having desired carbon chain characteristics. Exemplary fatty acid derivatives include for example, short and long chain alcohols, hydrocarbons, and fatty acid esters including waxes.

**Fermentation Broth:** Includes any medium which supports microorganism life (*i.e.* a microorganism that is actively metabolizing carbon). A fermentation medium usually contains a carbon source. The carbon source can be anything that can be utilized, with or without additional enzymes, by the microorganism for energy.

**Hydrocarbon:** includes chemical compounds that containing the elements carbon (C) and hydrogen (H). All hydrocarbons consist of a carbon backbone and atoms of hydrogen attached to that backbone. Sometimes, the term is used as a shortened form of the term "aliphatic hydrocarbon." There are essentially three types of hydrocarbons: (1) aromatic hydrocarbons, which have at least one aromatic ring; (2) saturated hydrocarbons, also known as alkanes, which lack double, triple or aromatic bonds; and (3) unsaturated hydrocarbons, which have one or more double or triple bonds between carbon atoms, are divided into: alkenes, alkynes, and dienes. Liquid geologically-extracted hydrocarbons are referred to as petroleum (literally "rock oil") or mineral oil, while gaseous geologic hydrocarbons are referred to as natural gas. All are significant sources of fuel and raw materials as a feedstock for the production of organic chemicals and are commonly found in the Earth's subsurface using the tools of petroleum geology. Oil reserves in sedimentary rocks are the principal source of hydrocarbons for the energy and chemicals industries. Hydrocarbons are of prime economic importance because they encompass the constituents of the major fossil fuels (coal, petroleum, natural gas, etc.) and biofuels, as well as plastics, waxes, solvents and oils.

**Isolated:** An “isolated” biological component (such as a nucleic acid molecule, protein, or cell) has been substantially separated or purified away from other biological components in which the component naturally occurs, such as other chromosomal and extrachromosomal DNA and RNA, and proteins. Nucleic acid molecules and proteins that have been “isolated” include nucleic acid molecules and proteins purified by standard purification methods. The term also embraces nucleic acid molecules and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acid molecules and proteins.

In one example, isolated refers to a naturally-occurring nucleic acid molecule that is not immediately contiguous with both of the sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally-occurring genome of the organism from which it is derived.

**Microorganism:** Includes prokaryotic and eukaryotic microbial species from the Domains Archaea, Bacteria and Eucarya, the latter including yeast and filamentous fungi, protozoa, algae, or higher Protista. The terms “microbial cells” and “microbes” are used interchangeably with the term microorganism.

**Nucleic Acid Molecule:** Encompasses both RNA and DNA molecules including, without limitation, cDNA, genomic DNA, and mRNA. Includes synthetic nucleic acid molecules, such as those that are chemically synthesized or recombinantly produced. The nucleic acid molecule can be double-stranded or single-stranded. Where single-stranded, the nucleic acid molecule can be the sense strand or the antisense strand. In addition, nucleic acid molecule can be circular or linear.

**Operably linked:** A first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein coding regions, in the same reading frame. Configurations of separate genes that are transcribed in tandem as a single messenger RNA are denoted as operons. Thus placing genes in

close proximity, for example in a plasmid vector, under the transcriptional regulation of a single promoter, constitutes a synthetic operon.

**ORF (open reading frame):** A series of nucleotide triplets (codons) coding for amino acids without any termination codons. These sequences are usually translatable into a peptide.

**Over-expressed:** When a gene is caused to be transcribed at an elevated rate compared to the endogenous transcription rate for that gene. In some examples, over-expression additionally includes an elevated rate of translation of the gene compared to the endogenous translation rate for that gene. Methods of testing for over-expression are well known in the art, for example transcribed RNA levels can be assessed using rtPCR and protein levels can be assessed using SDS page gel analysis.

**Purified:** The term purified does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified fatty acid derivative preparation, such as a wax, or a fatty acid ester preparation, is one in which the product is more concentrated than the product is in its environment within a cell. For example, a purified wax is one that is substantially separated from cellular components (nucleic acids, lipids, carbohydrates, and other peptides) that can accompany it. In another example, a purified wax preparation is one in which the wax is substantially-free from contaminants, such as those that might be present following fermentation.

In one example, a fatty acid ester is purified when at least about 50% by weight of a sample is composed of the fatty acid ester, for example when at least about 60%, 70%, 80%, 85%, 90%, 92%, 95%, 98%, or 99% or more of a sample is composed of the fatty acid ester. Examples of methods that can be used to purify a waxes, fatty alcohols, and fatty acid esters, include the methods described in Example 11 below.

**Recombinant:** A recombinant nucleic acid molecule or protein is one that has a sequence that is not naturally occurring, has a sequence that is made by an artificial combination of two otherwise separated segments of sequence, or both. This artificial combination can be achieved, for example, by chemical synthesis or by the artificial manipulation of isolated segments of nucleic acid molecules or

proteins, such as genetic engineering techniques. Recombinant is also used to describe nucleic acid molecules that have been artificially manipulated, but contain the same regulatory sequences and coding regions that are found in the organism from which the nucleic acid was isolated. A recombinant cell or microorganism is one that contains an exogenous nucleic acid molecule, such as a recombinant nucleic acid molecule.

**Release:** The movement of a compound from inside a cell (intracellular) to outside a cell (extracellular). The movement can be active or passive. When release is active it can be facilitated by one or more transporter peptides and in some examples it can consume energy. When release is passive, it can be through diffusion through the membrane and can be facilitated by continually collecting the desired compound from the extracellular environment, thus promoting further diffusion. Release of a compound can also be accomplished by lysing a cell.

**Surfactants:** Substances capable of reducing the surface tension of a liquid in which they are dissolved. They are typically composed of a water-soluble head and a hydrocarbon chain or tail. The water soluble group is hydrophilic and can be either ionic or nonionic, and the hydrocarbon chain is hydrophobic. Surfactants are used in a variety of products, including detergents and cleaners, and are also used as auxiliaries for textiles, leather and paper, in chemical processes, in cosmetics and pharmaceuticals, in the food industry and in agriculture. In addition, they can be used to aid in the extraction and isolation of crude oils which are found hard to access environments or as water emulsions.

There are four types of surfactants characterized by varying uses. Anionic surfactants have detergent-like activity and are generally used for cleaning applications. Cationic surfactants contain long chain hydrocarbons and are often used to treat proteins and synthetic polymers or are components of fabric softeners and hair conditioners. Amphoteric surfactants also contain long chain hydrocarbons and are typically used in shampoos. Non-ionic surfactants are generally used in cleaning products.

**Transformed or recombinant cell:** A cell into which a nucleic acid molecule has been introduced, such as an acyl-CoA synthase encoding nucleic acid molecule, for example by molecular biology techniques. Transformation

encompasses all techniques by which a nucleic acid molecule can be introduced into such a cell, including, but not limited to, transfection with viral vectors, conjugation, transformation with plasmid vectors, and introduction of naked DNA by electroporation, lipofection, and particle gun acceleration.

**Under conditions that permit product production:** Any fermentation conditions that allow a microorganism to produce a desired product, such as fatty acids, hydrocarbons, fatty alcohols, waxes, or fatty acid esters. Fermentation conditions usually include temperature ranges, levels of aeration, and media selection, which when combined allow the microorganism to grow. Exemplary mediums include broths or gels. Generally, the medium includes a carbon source such as glucose, fructose, cellulose, or the like that can be metabolized by the microorganism directly, or enzymes can be used in the medium to facilitate metabolizing the carbon source. To determine if culture conditions permit product production, the microorganism can be cultured for 24, 36, or 48 hours and a sample can be obtained and analyzed. For example, the cells in the sample or the medium in which the cells were grown can be tested for the presence of the desired product. When testing for the presence of a product assays, such as those provided in the Examples below, can be used.

**Vector:** A nucleic acid molecule as introduced into a cell, thereby producing a transformed cell. A vector can include nucleic acid sequences that permit it to replicate in the cell, such as an origin of replication. A vector can also include one or more selectable marker genes and other genetic elements known in the art.

**Wax:** A variety of fatty acid esters which form solids or pliable substances under an identified set of physical conditions. Fatty acid esters that are termed waxes generally have longer carbon chains than fatty acid esters that are not waxes. For example, a wax generally forms a pliable substance at room temperature.

### **Detailed Description**

#### **I. Production of fatty acid derivatives**

The host organism that exogenous DNA sequences are transformed into can be a modified host organism, such as an organism that has been modified to increase the production of acyl-ACP or acyl-CoA, reduce the catabolism of fatty acid



derivatives and intermediates, or to reduce feedback inhibition at specific points in the biosynthetic pathway. In addition to modifying the genes described herein additional cellular resources can be diverted to over produce fatty acids, for example the lactate, succinate and/or acetate pathways can be attenuated, and acetyl-CoA carboxylase (ACC) can be over expressed. The modifications to the production host described herein can be through genomic alterations, extrachromosomal expression systems, or combinations thereof. An overview of the pathway is provided in Figs. 1 and 2.

#### **A. Acetyl-CoA - Malonyl-CoA to Acyl-ACP**

Fatty acid synthase (FAS) is a group of peptides that catalyze the initiation and elongation of acyl chains (Marrakchi *et al.*, *Biochemical Society*, 30:1050-1055, 2002). The acyl carrier protein (ACP) along with the enzymes in the FAS pathway control the length, degree of saturation and branching of the fatty acids produced. Enzymes that can be included in FAS include AccABCD, FabD, FabH, FabG, FabA, FabZ, FabI, FabK, FabL, FabM, FabB, and FabF. Depending upon the desired product one or more of these genes can be attenuated or over-expressed.

For example, the fatty acid biosynthetic pathway in the production host uses the precursors acetyl-CoA and malonyl-CoA (Fig. 2). *E. coli* or other host organisms engineered to overproduce these components can serve as the starting point for subsequent genetic engineering steps to provide the specific output product (such as, fatty acid esters, hydrocarbons, fatty alcohols). Several different modifications can be made, either in combination or individually, to the host strain to obtain increased acetyl CoA/malonyl CoA/fatty acid and fatty acid derivative production. For example, to increase acetyl CoA production, a plasmid with *pdh*, *panK*, *aceEF*, (encoding the E1p dehydrogenase component and the E2p dihydrolipoamide acyltransferase component of the pyruvate and 2-oxoglutarate dehydrogenase complexes), *fabH* /*fabD*/*fabG*/*acpP*/*fabF*, and in some examples additional DNA encoding fatty-acyl-CoA reductases and aldehyde decarboxylases, all under the control of a constitutive, or otherwise controllable promoter, can be constructed. Exemplary Genbank accession numbers for these genes are: *pdh* (BAB34380, AAC73227, AAC73226), *panK* (also known as *coaA*, AAC76952),

*aceEF* (AAC73227, AAC73226), *fabH* (AAC74175), *fabD* (AAC74176), *fabG* (AAC74177), *acpP* (AAC74178), *fabF* (AAC74179).

Additionally, *fadE*, *gpsA*, *ldhA*, *pflb*, *adhE*, *pta*, *poxB*, *ackA*, and/or *ackB* can be knocked-out, or their expression levels can be reduced, in the engineered microorganism by transformation with conditionally replicative or non-replicative plasmids containing null or deletion mutations of the corresponding genes, or by substituting promoter or enhancer sequences. Exemplary Genbank accession numbers for these genes are; *fadE* (AAC73325), *gpsA* (AAC76632), *ldhA* (AAC74462), *pflb* (AAC73989), *adhE* (AAC74323), *pta* (AAC75357), *poxB* (AAC73958), *ackA* (AAC75356), and *ackB* (BAB81430).

The resulting engineered microorganisms can be grown in a desired environment, for example one with limited glycerol (less than 1% w/v in the culture medium). As such, these microorganisms will have increased acetyl-CoA production levels. Malonyl-CoA overproduction can be effected by engineering the microorganism as described above, with DNA encoding *accABCD* (acetyl CoA carboxylase, for example accession number AAC73296, EC 6.4.1.2) included in the plasmid synthesized *de novo*. Fatty acid overproduction can be achieved by further including DNA encoding lipase (for example Accessions numbers CAA89087, CAA98876) in the plasmid synthesized *de novo*.

In some examples, acetyl-CoA carboxylase (ACC) is over-expressed to increase the intracellular concentration thereof by at least 2-fold, such as at least 5-fold, or at least 10-fold, for example relative to native expression levels.

In addition, the *plsB* (for example Accession number AAC77011) D311E mutation can be used to remove limitations on the pool of acyl-CoA.

In addition, over-expression of an *sfa* gene (suppressor of FabA, for example Accession number AAN79592) can be included in the production host to increase production of monounsaturated fatty acids (Rock *et al.*, *J. Bacteriology* 178:5382-5387, 1996).

#### **B. Acyl-ACP to Fatty Acid**

To engineer a production host for the production of a homogeneous population of fatty acid derivatives, one or more endogenous genes can be attenuated or functionally deleted and one or more thioesterases can be expressed.

For example, C10 fatty acid derivatives can be produced by attenuating thioesterase C18 (for example accession numbers AAC73596 and P0ADA1 ), which uses C18:1-ACP and expressing thioesterase C10 (for example accession number Q39513), which uses C10-ACP. Thus, resulting in a relatively homogeneous population of fatty acid derivatives that have a carbon chain length of 10. In another example, C14 fatty acid derivatives can be produced by attenuating endogenous thioesterases that produce non-C14 fatty acids and expressing the thioesterase accession number Q39473 (which uses C14-ACP). In yet another example, C12 fatty acid derivatives can be produced by expressing thioesterases that use C12-ACP (for example accession number Q41635) and attenuating thioesterases that produce non-C12 fatty acids. Acetyl CoA, malonyl CoA, and fatty acid overproduction can be verified using methods known in the art, for example by using radioactive precursors, HPLC, and GC-MS subsequent to cell lysis.

**Table 1**  
**Thioesterases**

Accession Number	Source Organism	Gene	Preferential product produced
AAC73596	<i>E. coli</i>	<i>tesA</i> without leader sequence	C18:1
Q41635	<i>Umbellularia californica</i>	<i>fatB</i>	C12:0
Q39513;	<i>Cuphea hookeriana</i>	<i>fatB2</i>	C8:0 – C10:0
AAC49269	<i>Cuphea hookeriana</i>	<i>fatB3</i>	C14:0 - C16:0
Q39473	<i>Cinnamomum camphorum</i>	<i>fatB</i>	C14:0
CAA85388	<i>Arabidopsis thaliana</i>	<i>fatB</i> [M141T]*	C16:1
NP 189147; NP 193041	<i>Arabidopsis thaliana</i>	<i>fatA</i>	C18:1
CAC39106	<i>Bradyrhizobium japonicum</i>	<i>fatA</i>	C18:1
AAC72883	<i>Cuphea hookeriana</i>	<i>fatA</i>	C18:1

\*Mayer *et al.*, *BMC Plant Biology* 7:1-11, 2007.

### C. Fatty Acid to Acyl-CoA

Production hosts can be engineered using known peptides to produce fatty acids of various lengths. One method of making fatty acids involves increasing the expression of, or expressing more active forms of, one or more acyl-CoA synthase peptides (EC 2.3.1.86).

As used herein, acyl-CoA synthase includes peptides in enzyme classification number EC 2.3.1.86, as well as any other peptide capable of catalyzing the conversion of a fatty acid to acyl-CoA. Additionally, one of ordinary skill in the art will appreciate that some acyl-CoA synthase peptides will catalyze other reactions as well, for example some acyl-CoA synthase peptides will accept other substrates in addition to fatty acids. Such non-specific acyl-CoA synthase peptides are, therefore, also included. Acyl-CoA synthase peptide sequences are publicly available. Exemplary GenBank Accession Numbers are provided in Fig. 10.

#### **D. Acyl-CoA to fatty alcohol**

Production hosts can be engineered using known polypeptides to produce fatty alcohols from acyl-CoA. One method of making fatty alcohols involves increasing the expression of or expressing more active forms of fatty alcohol forming acyl-CoA reductase (FAR, EC 1.1.1.\*), or acyl-CoA reductases (EC 1.2.1.50) and alcohol dehydrogenase (EC 1.1.1.1). Hereinafter fatty alcohol forming acyl-CoA reductase (FAR, EC 1.1.1.\*), acyl-CoA reductases (EC 1.2.1.50) and alcohol dehydrogenase (EC 1.1.1.1) are collectively referred to as fatty alcohol forming peptides. In some examples all three of the fatty alcohol forming genes can be over expressed in a production host, and in yet other examples one or more of the fatty alcohol forming genes can be over-expressed.

As used herein, fatty alcohol forming peptides include peptides in enzyme classification numbers EC 1.1.1.\*, 1.2.1.50, and 1.1.1.1, as well as any other peptide capable of catalyzing the conversion of acyl-CoA to fatty alcohol. Additionally, one of ordinary skill in the art will appreciate that some fatty alcohol forming peptides will catalyze other reactions as well, for example some acyl-CoA reductase peptides will accept other substrates in addition to fatty acids. Such non-specific peptides are, therefore, also included. Fatty alcohol forming peptides sequences are publicly available. Exemplary GenBank Accession Numbers are provided in Fig. 10.

Fatty alcohols can also be described as hydrocarbon-based surfactants. For surfactant production the microorganism is modified so that it produces a surfactant from a renewable carbon source. Such a microorganism includes a first exogenous DNA sequence encoding a protein capable of converting a fatty acid to a fatty aldehyde and a second exogenous DNA sequence encoding a protein capable of converting a fatty aldehyde to an alcohol. In some examples, the first exogenous DNA sequence encodes a fatty acid reductase. In one embodiment, the second exogenous DNA sequence encodes mammalian microsomal aldehyde reductase or long-chain aldehyde dehydrogenase. In a further example, the first and second exogenous DNA sequences are from a multienzyme complex from *Arthrobacter AK 19*, *Rhodotorula glutinins*, *Acinobacter sp strain M-1*, or *Candida lipolytica*. In one embodiment, the first and second heterologous DNA sequences are from a multienzyme complex from *Acinobacter sp strain M-1* or *Candida lipolytica*.

Additional sources of heterologous DNA sequences encoding fatty acid to long chain alcohol converting proteins that can be used in surfactant production include, but are not limited to, *Mortierella alpina* (ATCC 32222), *Cryptococcus curvatus*, (also referred to as *Apiotricum curvatum*), *Alcanivorax jadensis* (T9T =DSM 12718 =ATCC 700854), *Acinetobacter sp. HO1-N*, (ATCC 14987) and *Rhodococcus opacus* (PD630 DSMZ 44193).

In one example, the fatty acid derivative is a saturated or unsaturated surfactant product having a carbon atom content limited to between 6 and 36 carbon atoms. In another example, the surfactant product has a carbon atom content limited to between 24 and 32 carbon atoms.

Appropriate hosts for producing surfactants can be either eukaryotic or prokaryotic microorganisms. Exemplary hosts include *Arthrobacter AK 19*, *Rhodotorula glutinins*, *Acinobacter sp strain M-1*, *Arabidopsis thaliana*, or *Candida lipolytica*, *Saccharomyces cerevisiae*, and *E. coli* engineered to express acetyl CoA carboxylase. Hosts which demonstrate an innate ability to synthesize high levels of surfactant precursors in the form of lipids and oils, such as *Rhodococcus opacus*, *Arthrobacter AK 19*, *Rhodotorula glutinins* *E. coli* engineered to express acetyl CoA carboxylase, and other oleaginous bacteria, yeast, and fungi can also be used.

#### **E. Fatty Alcohols to Fatty Esters**

Production hosts can be engineered using known polypeptides to produce fatty esters of various lengths. One method of making fatty esters includes increasing the expression of, or expressing more active forms of, one or more alcohol *O*-acetyltransferase peptides (EC 2.3.1.84). These peptides catalyze the reaction of acetyl-CoA and an alcohol to form CoA and an acetic ester. In some examples the alcohol *O*-acetyltransferase peptides can be expressed in conjunction with selected thioesterase peptides, FAS peptides and fatty alcohol forming peptides, thus, allowing the carbon chain length, saturation and degree of branching to be controlled. In some cases the *bkd* operon can be coexpressed to enable branched fatty acid precursors to be produced.

As used herein, alcohol *O*-acetyltransferase peptides include peptides in enzyme classification number EC 2.3.1.84, as well as any other peptide capable of catalyzing the conversion of acetyl-CoA and an alcohol to form CoA and an acetic ester. Additionally, one of ordinary skill in the art will appreciate that alcohol *O*-acetyltransferase peptides will catalyze other reactions as well, for example some alcohol *O*-acetyltransferase peptides will accept other substrates in addition to fatty alcohols or acetyl-CoA thioester i.e such as other alcohols and other acyl-CoA thioesters. Such non-specific or divergent specificity alcohol *O*-acetyltransferase peptides are, therefore, also included. Alcohol *O*-acetyltransferase peptide sequences are publicly available. Exemplary GenBank Accession Numbers are provided in Fig. 10. Assays for characterizing the activity of a particular alcohol *O*-acetyltransferase peptides are well known in the art. Engineered *O*-acetyltransferases and *O*-acyltransferases can be also created that have new activities and specificities for the donor acyl group or acceptor alcohol moiety. Engineered enzymes could be generated through rational and evolutionary approaches well documented in the art.

#### **F. Acyl-CoA to Fatty Esters (biodiesels and waxes)**

Production hosts can be engineered using known peptides to produce fatty acid esters from acyl-CoA and alcohols. In some examples the alcohols are provided in the fermentation media and in other examples the production host can provide the alcohol as described herein. One of ordinary skill in the art will appreciate that structurally, fatty acid esters have an A and a B side. As described

herein, the A side of the ester is used to describe the carbon chain contributed by the alcohol, and the B side of the ester is used to describe the carbon chain contributed by the acyl-CoA. Either chain can be saturated or unsaturated, branched or unbranched. The production host can be engineered to produce fatty alcohols or short chain alcohols. The production host can also be engineered to produce specific acyl-CoA molecules. As used herein fatty acid esters are esters derived from a fatty acyl-thioester and an alcohol, wherein the A side and the B side of the ester can vary in length independently. Generally, the A side of the ester is at least 1, 2, 3, 4, 5, 6, 7, or 8 carbons in length, while the B side of the ester is 8, 10, 12, 14, 16, 18, 20, 22, 24, or 26 carbons in length. The A side and the B side can be straight chain or branched, saturated or unsaturated.

The production of fatty esters, including waxes from acyl-CoA and alcohols can be engineered using known polypeptides. As used herein waxes are long chain fatty acid esters, wherein the A side and the B side of the ester can vary in length independently. Generally, the A side of the ester is at least 8, 10, 12, 14, 16, 18, 20, 22, 24, or 26 carbons in length. Similarly the B side of the ester is at least 8, 10, 12, 14, 16, 18, 20, 22, 24, or 26 carbons in length. The A side and the B side can be mono-, di-, tri- unsaturated. The production of fatty esters, including waxes from acyl-CoA and alcohols can be engineered using known polypeptides. One method of making fatty esters includes increasing the expression of or expressing more active forms of one or more wax synthases (EC 2.3.1.75).

As used herein, wax synthases includes peptides in enzyme classification number EC 2.3.1.75, as well as any other peptide capable of catalyzing the conversion of an acyl-thioester to fatty esters. Additionally, one of ordinary skill in the art will appreciate that some wax synthase peptides will catalyze other reactions as well, for example some wax synthase peptides will accept short chain acyl-CoAs and short chain alcohols to produce fatty esters. Such non-specific wax synthases are, therefore, also included. Wax synthase peptide sequences are publicly available. Exemplary GenBank Accession Numbers are provided in Fig. 10. Methods to identify wax synthase activity are provided in U.S. patent number 7,118,896, which is herein incorporated by reference.

In particular examples, if the desired product is a fatty ester based biofuel, the microorganism is modified so that it produces a fatty ester generated from a renewable energy source. Such a microorganism includes an exogenous DNA sequence encoding a wax ester synthase that is expressed so as to confer upon said microorganism the ability to synthesize a saturated, unsaturated, or branched fatty ester from a renewable energy source. In some embodiments, the wax ester synthesis proteins include, but are not limited to, fatty acid elongases, acyl-CoA reductases, acyltransferases or wax synthases, fatty acyl transferases, diacylglycerol acyltransferases, acyl-coA wax alcohol acyltransferases, bifunctional wax ester synthase/acyl-CoA:diacylglycerol acyltransferase selected from a multienzyme complex from *Simmondsia chinensis*, *Acinetobacter* sp. strain ADP1 (formerly *Acinetobacter calcoaceticus* ADP1), *Pseudomonas aeruginosa*, *Fundibacter jadensis*, *Arabidopsis thaliana*, or *Alkaligenes eutrophus*. In one embodiment, the fatty acid elongases, acyl-CoA reductases or wax synthases are from a multienzyme complex from *Alkaligenes eutrophus* and other organisms known in the literature to produce wax and fatty acid esters.

Additional sources of heterologous DNA encoding wax synthesis proteins useful in fatty ester production include, but are not limited to, *Mortierella alpina* (for example ATCC 32222), *Cryptococcus curvatus*, (also referred to as *Apiotricum curvatum*), *Alcanivorax jadensis* (for example T9T = DSM 12718 = ATCC 700854), *Acinetobacter* sp. HO1-N, (for example ATCC 14987) and *Rhodococcus opacus* (for example PD630, DSMZ 44193).

The methods of described herein permit production of fatty esters of varied length. In one example, the fatty ester product is a saturated or unsaturated fatty ester product having a carbon atom content between 24 and 46 carbon atoms. In one embodiment, the fatty ester product has a carbon atom content between 24 and 32 carbon atoms. In another embodiment the fatty ester product has a carbon content of 14 and 20 carbons. In another embodiment the fatty ester is the methyl ester of C18:1. In another embodiment the fatty acid ester is the ethyl ester of C16:1. In another embodiment the fatty ester is the methyl ester of C16:1. In another embodiment the fatty acid ester is octadecyl ester of octanol.



Useful hosts for producing fatty esters can be either eukaryotic or prokaryotic microorganisms. In some embodiments such hosts include, but are not limited to, *Saccharomyces cerevisiae*, *Candida lipolytica*, *E. coli* *Arthrobacter AK 19*, *Rhodotorula glutinins*, *Acinetobacter sp strain M-1*, *Candida lipolytica* and other oleaginous microorganisms.

In one example the wax ester synthase from *Acinetobacter sp.* ADP1 at locus AAO17391 (described in Kalscheuer and Steinbuchel, *J. Biol. Chem.* 278:8075-8082, 2003, herein incorporated by reference) is used. In another example the wax ester synthase from *Simmondsia chinensis*, at locus AAD38041 is used.

Optionally a wax ester exporter such as a member of the FATP family can be used to facilitate the release of waxes or esters into the extracellular environment. One example of a wax ester exporter that can be used is fatty acid (long chain) transport protein CG7400-PA, isoform A from *Drosophila melanogaster*, at locus NP\_524723.

#### **G. Acyl-ACP, Acyl-CoA to Hydrocarbon**

A diversity of microorganisms are known to produce hydrocarbons, such as alkanes, olefins, and isoprenoids. Many of these hydrocarbons are derived from fatty acid biosynthesis. The production of these hydrocarbons can be controlled by controlling the genes associated with fatty acid biosynthesis in the native hosts. For example, hydrocarbon biosynthesis in the algae *Botryococcus braunii* occurs through the decarbonylation of fatty aldehydes. The fatty aldehydes are produced by the reduction of fatty acyl – thioesters by fatty acyl-CoA reductase. Thus, the structure of the final alkanes can be controlled by engineering *B. braunii* to express specific genes, such as thioesterases, which control the chain length of the fatty acids being channeled into alkane biosynthesis. Expressing the enzymes that result in branched chain fatty acid biosynthesis in *B. braunii* will result in the production of branched chain alkanes. Introduction of genes effecting the production of desaturation of fatty acids will result in the production of olefins. Further combinations of these genes can provide further control over the final structure of the hydrocarbons produced. To produce higher levels of the native or engineered hydrocarbons, the genes involved in the biosynthesis of fatty acids and their precursors or the degradation to other products can be expressed, overexpressed, or

attenuated. Each of these approaches can be applied to the production of alkanes in *Vibrio furnissi* M1 and its functional homologues, which produces alkanes through the reduction of fatty alcohols (see above for the biosynthesis and engineering of fatty alcohol production). Each of these approaches can also be applied to the production of the olefins produced by many strains of *Micrococcus leuteus*, *Stenotrophomonas maltophilia*, *Jeogalicoccus sp.* (ATCC8456), and related microorganisms. These microorganisms produce long chain internal olefins that are derived from the head to head condensation of fatty acid precursors. Controlling the structure and level of the fatty acid precursors using the methods described herein will result in formation of olefins of different chain length, branching, and level of saturation.

Hydrocarbons can also be produced using evolved oxido/reductases for the reduction of primary alcohols. Primary fatty alcohols are known to be used to produce alkanes in microorganisms such as *Vibrio furnissii* M1 (Myong-Ok, *J. Bacteriol.*, 187:1426-1429, 2005). An NAD(P)H dependent oxido/reductase is the responsible catalyst. Synthetic NAD(P)H dependent oxidoreductases can be produced through the use of evolutionary engineering and be expressed in production hosts to produce fatty acid derivatives. One of ordinary skill in the art will appreciate that the process of "evolving" a fatty alcohol reductase to have the desired activity is well known (Kolkman and Stemmer *Nat Biotechnol.* 19:423-8, 2001, Ness *et al.*, *Adv Protein Chem.* 55:261-92, 2000, Minshull and Stemmer *Curr Opin Chem Biol.* 3:284-90, 1999, Huisman and Gray *Curr Opin Biotechnol.* Aug;13:352-8, 2002, and see U.S. patent application 2006/0195947). A library of NAD(P)H dependent oxidoreductases is generated by standard methods, such as error prone PCR, site-specific random mutagenesis, site specific saturation mutagenesis, or site directed specific mutagenesis. Additionally, a library can be created through the "shuffling" of naturally occurring NAD(P)H dependent oxidoreductase encoding sequences. The library is expressed in a suitable host, such as *E. coli*. Individual colonies expressing a different member of the oxido/reductase library is then analyzed for its expression of an oxido/reductase that can catalyze the reduction of a fatty alcohol. For example, each cell can be assayed as a whole cell bioconversion, a cell extract, a permeabilized cell, or a purified enzyme. Fatty

alcohol reductases are identified by the monitoring the fatty alcohol dependent oxidation of NAD(P)H spectrophotometrically or fluorometrically. Production of alkanes is monitored by GC/MS, TLC, or other methods. An oxido/reductase identified in this manner is used to produce alkanes, alkenes, and related branched hydrocarbons. This is achieved either *in vitro* or *in vivo*. The later is achieved by expressing the evolved fatty alcohol reductase gene in an organism that produces fatty alcohols, such as those described herein. The fatty alcohols act as substrates for the alcohol reductase which would produce alkanes. Other oxidoreductases can be also engineered to catalyze this reaction, such as those that use molecular hydrogen, glutathione, FADH, or other reductive coenzymes.

## **II. Genetic Engineering of Production Strain to increase Fatty Acid Derivative Production**

Heterologous DNA sequences involved in a biosynthetic pathway for the production of fatty acid derivatives can be introduced stably or transiently into a host cell using techniques well known in the art for example electroporation, calcium phosphate precipitation, DEAE-dextran mediated transfection, liposome-mediated transfection, conjugation, transduction, and the like. For stable transformation, a DNA sequence can further include a selectable marker, such as, antibiotic resistance, for example resistance to neomycin, tetracycline, chloramphenicol, kanamycin, genes that complement auxotrophic deficiencies, and the like.

Various embodiments of this disclosure utilize an expression vector that includes a heterologous DNA sequence encoding a protein involved in a metabolic or biosynthetic pathway. Suitable expression vectors include, but are not limited to, viral vectors, such as baculovirus vectors, phage vectors, such as bacteriophage vectors, plasmids, phagemids, cosmids, fosmids, bacterial artificial chromosomes, viral vectors (*e.g.* viral vectors based on vaccinia virus, poliovirus, adenovirus, adeno-associated virus, SV40, herpes simplex virus, and the like), P1-based artificial chromosomes, yeast plasmids, yeast artificial chromosomes, and any other vectors specific for specific hosts of interest (such as *E. coli*, *Pseudomonas putida* and *Saccharomyces cerevisiae*).

Useful expression vectors can include one or more selectable marker genes

to provide a phenotypic trait for selection of transformed host cells. The selectable marker gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selectable marker gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.*, ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, *e.g.*, the gene encoding D-alanine racemase for *Bacilli*. In alternative embodiments, the selectable marker gene is one that encodes dihydrofolate reductase or confers neomycin resistance (for use in eukaryotic cell culture), or one that confers tetracycline or ampicillin resistance (for use in a prokaryotic host cell, such as *E. coli*).

The biosynthetic pathway gene product-encoding DNA sequence in the expression vector is operably linked to an appropriate expression control sequence, (promoters, enhancers, and the like) to direct synthesis of the encoded gene product. Such promoters can be derived from microbial or viral sources, including CMV and SV40. Depending on the host/vector system utilized, any of a number of suitable transcription and translation control elements, including constitutive and inducible promoters, transcription enhancer elements, transcription terminators, *etc.* can be used in the expression vector (see *e.g.*, Bitter *et al.*, *Methods in Enzymology*, 153:516- 544, 1987).

Suitable promoters for use in prokaryotic host cells include, but are not limited to, promoters capable of recognizing the T4, T3, Sp6 and T7 polymerases, the P<sub>R</sub> and P<sub>L</sub> promoters of bacteriophage lambda, the trp, recA, heat shock, and lacZ promoters of *E. coli*, the alpha-amylase and the sigma-specific promoters of *B. subtilis*, the promoters of the bacteriophages of *Bacillus*, *Streptomyces* promoters, the int promoter of bacteriophage lambda, the bla promoter of the beta-lactamase gene of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene. Prokaryotic promoters are reviewed by Glick, *J. Ind. Microbiol.* 1:277, 1987; Watson *et al.*, MOLECULAR BIOLOGY OF THE GENE, 4th Ed., Benjamin Cummins (1987); and Sambrook *et al.*, *supra*.

Non-limiting examples of suitable eukaryotic promoters for use within a

eukaryotic host are viral in origin and include the promoter of the mouse metallothionein I gene (Hamer *et al.*, *J. Mol. Appl. Gen.* 1:273, 1982); the TK promoter of Herpes virus (McKnight, *Cell* 31:355, 1982); the SV40 early promoter (Benoist *et al.*, *Nature (London)* 290:304, 1981); the Rous sarcoma virus promoter; the cytomegalovirus promoter (Foecking *et al.*, *Gene* 45:101, 1980); the yeast gal4 gene promoter (Johnston, *et al.*, *PNAS (USA)* 79:6971, 1982; Silver, *et al.*, *PNAS (USA)* 81:5951, 1984); and the IgG promoter (Orlandi *et al.*, *PNAS (USA)* 86:3833, 1989).

The microbial host cell can be genetically modified with a heterologous DNA sequence encoding a biosynthetic pathway gene product that is operably linked to an inducible promoter. Inducible promoters are well known in the art. Suitable inducible promoters include, but are not limited to promoters that are affected by proteins, metabolites, or chemicals. These include: a bovine leukemia virus promoter, a metallothionein promoter, a dexamethasone-inducible MMTV promoter, a SV40 promoter, a MRP polIII promoter, a tetracycline-inducible CMV promoter (such as the human immediate-early CMV promoter) as well as those from the trp and lac operons.

In some examples a genetically modified host cell is genetically modified with a heterologous DNA sequence encoding a biosynthetic pathway gene product that is operably linked to a constitutive promoter. Suitable constitutive promoters are known in the art and include, constitutive adenovirus major late promoter, a constitutive MPSV promoter, and a constitutive CMV promoter.

In some examples a modified host cell is one that is genetically modified with an exogenous DNA sequence encoding a single protein involved in a biosynthesis pathway. In other embodiments, a modified host cell is one that is genetically modified with exogenous DNA sequences encoding two or more proteins involved in a biosynthesis pathway -- for example, the first and second enzymes in a biosynthetic pathway.

Where the host cell is genetically modified to express two or more proteins involved in a biosynthetic pathway, those DNA sequences can each be contained in a single or in separate expression vectors. When those DNA sequences are contained in a single expression vector, in some embodiments, the nucleotide

sequences will be operably linked to a common control element (*e.g.*, a promoter), *e.g.*, the common control element controls expression of all of the biosynthetic pathway protein-encoding DNA sequences in the single expression vector.

When a modified host cell is genetically modified with heterologous DNA sequences encoding two or more proteins involved in a biosynthesis pathway, one of the DNA sequences can be operably linked to an inducible promoter, and one or more of the DNA sequences can be operably linked to a constitutive promoter.

In some embodiments, the intracellular concentration (*e.g.*, the concentration of the intermediate in the genetically modified host cell) of the biosynthetic pathway intermediate can be increased to further boost the yield of the final product. The intracellular concentration of the intermediate can be increased in a number of ways, including, but not limited to, increasing the concentration in the culture medium of a substrate for a biosynthetic pathway; increasing the catalytic activity of an enzyme that is active in the biosynthetic pathway; increasing the intracellular amount of a substrate (*e.g.*, a primary substrate) for an enzyme that is active in the biosynthetic pathway; and the like.

In some examples the fatty acid derivative or intermediate is produced in the cytoplasm of the cell. The cytoplasmic concentration can be increased in a number of ways, including, but not limited to, binding of the fatty acid to coenzyme A to form an acyl-CoA thioester. Additionally, the concentration of acyl-CoAs can be increased by increasing the biosynthesis of CoA in the cell, such as by over-expressing genes associated with pantothenate biosynthesis (*panD*) or knocking out the genes associated with glutathione biosynthesis (glutathione synthase).

### **III. Carbon chain characteristics**

Using the teachings provided herein a range of products can be produced. These products include hydrocarbons, fatty alcohols, fatty acid esters, and waxes. Some of these products are useful as biofuels and specialty chemicals. These products can be designed and produced in microorganisms. The products can be produced such that they contain branch points, levels of saturation, and carbon chain length, thus, making these products desirable starting materials for use in many

applications (Fig. 10 provides a description of the various enzymes that can be used alone or in combination to make various fatty acid derivatives).

In other examples, the expression of exogenous FAS genes originating from different species or engineered variants can be introduced into the host cell to result in the biosynthesis of fatty acid metabolites structurally different (in length, branching, degree of unsaturation, etc.) as that of the native host. These heterologous gene products can be also chosen or engineered so that they are unaffected by the natural complex regulatory mechanisms in the host cell and, therefore, function in a manner that is more controllable for the production of the desired commercial product. For example the FAS enzymes from *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Streptomyces spp*, *Ralstonia*, *Rhodococcus*, *Corynebacteria*, *Brevibacteria*, *Mycobacteria*, oleaginous yeast, and the like can be expressed in the production host.

One of ordinary skill in the art will appreciate that when a production host is engineered to produce a fatty acid from the fatty acid biosynthetic pathway that contains a specific level of unsaturation, branching, or carbon chain length the resulting engineered fatty acid can be used in the production of the fatty acid derivatives. Hence, fatty acid derivatives generated from the production host can display the characteristics of the engineered fatty acid. For example, a production host can be engineered to make branched, short chain fatty acids, and then using the teachings provided herein relating to fatty alcohol production (i.e. including alcohol forming enzymes such as FAR) the production host produce branched, short chain fatty alcohols. Similarly, a hydrocarbon can be produced by engineering a production host to produce a fatty acid having a defined level of branching, unsaturation, and/or carbon chain length, thus, producing a homogenous hydrocarbon population. Moreover, when an unsaturated alcohol, fatty acid ester, or hydrocarbon is desired the fatty acid biosynthetic pathway can be engineered to produce low levels of saturated fatty acids and an additional desaturase can be expressed to lessen the saturated product production.

#### **A. Saturation**

Production hosts can be engineered to produce unsaturated fatty acids by engineering the production host to over-express *fabB*, or by growing the production host at low temperatures (for example less than 37°C). *FabB* has preference to cis- $\delta^3$ decenoyl-ACP and results in unsaturated fatty acid production in *E. coli*. Over-expression of *FabB* resulted in the production of a significant percentage of unsaturated fatty acids (de Mendoza *et al.*, *J. Biol. Chem.*, 258:2098-101, 1983). These unsaturated fatty acids can then be used as intermediates in production hosts that are engineered to produce fatty acid derivatives, such as fatty alcohols, esters, waxes, olefins, alkanes, and the like. One of ordinary skill in the art will appreciate that by attenuating *fabA*, or over-expressing *FabB* and expressing specific thioesterases (described below), unsaturated fatty acid derivatives having a desired carbon chain length can be produced. Alternatively, the repressor of fatty acid biosynthesis, *FabR* (Genbank accession NP\_418398), can be deleted, which will also result in increased unsaturated fatty acid production in *E. coli* (Zhang *et al.*, *J. Biol. Chem.* 277:pp. 15558, 2002.). Further increase in unsaturated fatty acids may be achieved by over-expression of *FabM* (trans-2, cis-3-decenoyl-ACP isomerase, Genbank accession DAA05501) and controlled expression of *FabK* (trans-2-enoyl-ACP reductase II, Genbank accession NP\_357969) from *Streptococcus pneumoniae* (Marrakchi *et al.*, *J. Biol. Chem.* 277: 44809, 2002), while deleting *E. coli* *Fab I* ((trans-2-enoyl-ACP reductase, Genbank accession NP\_415804). Additionally, to increase the percentage of unsaturated fatty acid esters, the microorganism can also have *fabB* (encoding  $\beta$ -ketoacyl-ACP synthase I, Accessions: BAA16180, EC:2.3.1.41), *Sfa* (encoding a suppressor of *fabA*, Accession: AAC44390) and *gnsA* and *gnsB* (both encoding *secG* null mutant suppressors, a.k.a. cold shock proteins, Accession: ABD18647.1, AAC74076.1) over-expressed.

In some examples, the endogenous *fabF* gene can be attenuated, thus, increasing the percentage of palmitoleate (C16:1) produced.

#### **B. Branching including cyclic moieties**

Fatty acid derivatives can be produced that contain branch points, cyclic moieties, and combinations thereof, using the teachings provided herein.

Microorganisms that naturally produce straight fatty acids (sFAs) can be engineered to produce branched chain fatty acids (brFAs) by expressing one or more



exogenous nucleic acid sequences. For example, *E. coli* naturally produces straight fatty acids (sFAs). To engineer *E. coli* to produce brFAs, several genes can be introduced and expressed that provide branched precursors (bkd operon) and allow initiation of fatty acid biosynthesis from branched precursors (fabH). Additionally, the organism can express genes for the elongation of brFAs (e.g. ACP, FabF) and/or deleting the corresponding *E. coli* genes that normally lead to sFAs and would compete with the introduced genes (e.g. FabH, FabF).

The branched acyl-CoAs 2-methyl-buturyl-CoA, isovaleryl-CoA and isobutyryl-CoA are the precursors of brFA. In most brFA-containing microorganisms they are synthesized in two steps (described in detail below) from branched amino acids (isoleucine, leucine and valine) (Kadena, *Microbiol. Rev.* 55: pp. 288, 1991). To engineer a microorganism to produce brFAs, or to overproduce brFAs, expression or over-expression of one or more of the enzymes in these two steps can be engineered. For example, in some instances the production host may have an endogenous enzyme that can accomplish one step and therefore, only enzymes involved in the second step need to be expressed recombinantly.

The first step in forming branched fatty acids is the production of the corresponding  $\alpha$ -keto acids by a branched-chain amino acid aminotransferase. *E. coli* has such an enzyme, IlvE (EC 2.6.1.42; Genbank accession YP\_026247). In some examples, a heterologous branched-chain amino acid aminotransferase may not be expressed. However, *E. coli* IlvE or any other branched-chain amino acid aminotransferase, e.g. ilvE from *Lactococcus lactis* (Genbank accession AAF34406), ilvE from *Pseudomonas putida* (Genbank accession NP\_745648) or ilvE from *Streptomyces coelicolor* (Genbank accession NP\_629657) can be over-expressed in a host microorganism, should the aminotransferase reaction turn out to be rate limiting in brFA biosynthesis in the host organism chosen for fatty acid derivative production.

The second step, the oxidative decarboxylation of the  $\alpha$ -ketoacids to the corresponding branched-chain acyl-CoA, is catalyzed by a branched-chain  $\alpha$ -keto acid dehydrogenase complexes (bkd; EC 1.2.4.4.) (Denoya *et al. J. Bacteriol.* 177:pp. 3504, 1995), which consist of E1 $\alpha/\beta$  (decarboxylase), E2 (dihydrolipoyl transacylase) and E3 (dihydrolipoyl dehydrogenase) subunits and are similar to

pyruvate and  $\alpha$ -ketoglutarate dehydrogenase complexes. **Table 2** shows potential *bkd* genes from several microorganisms, that can be expressed in a production host to provide branched-chain acyl-CoA precursors. Basically, every microorganism that possesses brFAs and/or grows on branched-chain amino acids can be used as a source to isolate *bkd* genes for expression in production hosts such as, for example, *E. coli*. Furthermore, *E. coli* has the E3 component (as part of its pyruvate dehydrogenase complex; lpd, EC 1.8.1.4, Genbank accession NP\_414658), it can therefore, be sufficient to only express the *E1*  $\alpha/\beta$  and *E2* *bkd* genes.

**Table 2**  
**Bkd genes from selected microorganisms**

Organism	Gene	Genbank Accession #
<i>Streptomyces coelicolor</i>	<i>bkdA1</i> (E1 $\alpha$ )	NP_628006
	<i>bkdB1</i> (E1 $\beta$ )	NP_628005
	<i>bkdC1</i> (E2)	NP_638004
<i>Streptomyces coelicolor</i>	<i>bkdA2</i> (E1 $\alpha$ )	NP_733618
	<i>bkdB2</i> (E1 $\beta$ )	NP_628019
	<i>bkdC2</i> (E2)	NP_628018
<i>Streptomyces avermitilis</i>	<i>bkdA</i> (E1a)	BAC72074
	<i>bkdB</i> (E1b)	BAC72075
	<i>bkdC</i> (E2)	BAC72076
<i>Streptomyces avermitilis</i>	<i>bkdF</i> (E1 $\alpha$ )	BAC72088
	<i>bkdG</i> (E1 $\beta$ )	BAC72089
	<i>bkdH</i> (E2)	BAC72090
<i>Bacillus subtilis</i>	<i>bkdAA</i> (E1 $\alpha$ )	NP_390288
	<i>bkdAB</i> (E1 $\beta$ )	NP_390288
	<i>bkdB</i> (E2)	NP_390288
<i>Pseudomonas putida</i>	<i>bkdA1</i> (E1 $\alpha$ )	AAA65614
	<i>bkdA2</i> (E1 $\beta$ )	AAA65615
	<i>bkdC</i> (E2)	AAA65617

In another example, isobutyryl-CoA can be made in a production host, for example in *E. coli* through the coexpression of a crotonyl-CoA reductase (Ccr, EC 1.1.1.9) and isobutyryl-CoA mutase (large subunit IcmA, EC 5.4.99.2; small subunit IcmB, EC 5.4.99.13 ) (Han and Reynolds *J. Bacteriol.* 179:pp. 5157, 1997). Crotonyl-CoA is an intermediate in fatty acid biosynthesis in *E. coli* and other microorganisms. Examples for *ccr* and *icm* genes from selected microorganisms are given in **Table 3**.

**Table 3**  
***Ccr* and *icm* genes from selected microorganisms**

Organism	Gene	Genbank Accession #
<i>Streptomyces coelicolor</i>	<i>ccr</i>	NP_630556
	<i>icmA</i>	NP_629554
	<i>icmB</i>	NP_630904
<i>Streptomyces cinnamonensis</i>	<i>ccr</i>	AAD53915
	<i>icmA</i>	AAC08713
	<i>icmB</i>	AJ246005

In addition to expression of the *bkd* genes (see above), the initiation of brFA biosynthesis utilizes  $\beta$ -ketoacyl-acyl-carrier-protein synthase III (FabH, EC 2.3.1.41) with specificity for branched chain acyl CoAs (Li *et al.* *J. Bacteriol.* 187:pp. 3795, 2005). Examples of such FabHs are listed in **Table 4**. *FabH* genes that are involved in fatty acid biosynthesis of any brFA-containing microorganism can be expressed in a production host. The Bkd and FabH enzymes from production hosts that do not naturally make brFA may not support brFA production and therefore, Bkd and FabH can be expressed recombinantly. Similarly, the endogenous level of Bkd and FabH production may not be sufficient to produce brFA, therefore, they can be over-expressed. Additionally, other components of fatty acid biosynthesis machinery can be expressed such as acyl carrier proteins (ACPs) and  $\beta$ -ketoacyl-acyl-carrier-protein synthase II candidates are acyl carrier proteins (ACPs) and  $\beta$ -ketoacyl-acyl-carrier-protein synthase II (*fabF*, EC 2.3.1.41) (candidates are listed in **Table 4**). In addition to expressing these genes, some genes in the endogenous fatty acid biosynthesis pathway may be attenuated in the production host. For example, in *E. coli* the most likely candidates to interfere with brFA biosynthesis are *fabH* (Genbank accession # NP\_415609) and/or *fabF* genes (Genbank accession # NP\_415613).

As mentioned above, through the combination of expressing genes that support brFA synthesis and alcohol synthesis branched chain alcohols can be produced. For example, when an alcohol reductase such as Acr1 from *Acinetobacter baylyi* ADP1 is coexpressed with a *bkd* operon, *E. coli* can synthesize

isopentanol, isobutanol or 2-methyl butanol. Similarly, when Acr1 is coexpressed with *ccr/icm* genes, *E. coli* can synthesize isobutanol.

In order to convert a production host such as *E. coli* into an organism capable of synthesizing  $\omega$ -cyclic fatty acids (cyFAs), several genes need to be introduced and expressed that provide the cyclic precursor cyclohexylcarbonyl-CoA (Cropp *et al. Nature Biotech.* 18:pp. 980, 2000). The genes listed in **Table 4** (*fabH*, *ACP* and *fabF*) can then be expressed to allow initiation and elongation of  $\omega$ -cyclic fatty acids. Alternatively, the homologous genes can be isolated from microorganisms that make cyFAs and expressed in *E. coli*.

**Table 4**

***FabH*, *ACP* and *fabF* genes from selected microorganisms with brFAs**

Organism	Gene	Genbank Accession #
<i>Streptomyces coelicolor</i>	<i>fabH1</i> <i>ACP</i> <i>fabF</i>	NP_626634 NP_626635 NP_626636
<i>Streptomyces avermitilis</i>	<i>fabH3</i> <i>fabC3 (ACP)</i> <i>fabF</i>	NP_823466 NP_823467 NP_823468
<i>Bacillus subtilis</i>	<i>fabH_A</i> <i>fabH_B</i> <i>ACP</i> <i>fabF</i>	NP_389015 NP_388898 NP_389474 NP_389016
<i>Stenotrophomonas maltophilia</i>	SmalDRAFT_0818 ( <i>FabH</i> ) SmalDRAFT_0821 ( <i>ACP</i> ) SmalDRAFT_0822 ( <i>FabF</i> )	ZP_01643059 ZP_01643063 ZP_01643064
<i>Legionella pneumophila</i>	<i>FabH</i> <i>ACP</i> <i>fabF</i>	YP_123672 YP_123675 YP_123676

Expression of the following genes are sufficient to provide cyclohexylcarbonyl-CoA in *E. coli*: *ansJ*, *ansK*, *ansL*, *chcA* and *ansM* from the ansatrienin gene cluster of *Streptomyces collinus* (Chen *et al.*, *Eur. J. Biochem.* 261:pp. 1999, 1999) or *plmJ*, *plmK*, *plmL*, *chcA* and *plmM* from the phoslactomycin B gene cluster of *Streptomyces* sp. HK803 (Palaniappan *et al.*, *J. Biol. Chem.* 278:pp. 35552, 2003) together with the *chcB* gene (Patton *et al. Biochem.*, 39:pp. 7595, 2000) from *S. collinus*, *S. avermitilis* or *S. coelicolor* (see **Table 5** for Genbank accession numbers).

**Table 5**  
**Genes for the synthesis of cyclohexylcarbonyl-CoA**

Organism	Gene	Genbank Accession #
<i>Streptomyces collinus</i>	<i>ansJK</i> <i>ansL</i> <i>chcA</i> <i>ansL</i> <i>chcB</i>	U72144*    AF268489
<i>Streptomyces</i> sp. HK803	<i>pmlJK</i> <i>pmlL</i> <i>chcA</i> <i>pmlM</i>	AAQ84158 AAQ84159 AAQ84160 AAQ84161
<i>Streptomyces coelicolor</i>	<i>chcB/caiD</i>	NP_629292
<i>Streptomyces avermitilis</i>	<i>chcB/caiD</i>	NP_629292

Only *chcA* is annotated in Genbank entry U72144, *ansJKLM* are according to Chen *et al.* (*Eur. J. Biochem.* 261:pp. 1999, 1999)

The genes listed in **Table 4** (*fabH*, *ACP* and *fabF*) are sufficient to allow initiation and elongation of  $\omega$ -cyclic fatty acids, because they can have broad substrate specificity. In the event that coexpression of any of these genes with the *ansJKLM/chcAB* or *pmlJKLM/chcAB* genes from **Table 5** does not yield cyFAs, *fabH*, *ACP* and/or *fabF* homologs from microorganisms that make cyFAs can be isolated (e.g. by using degenerate PCR primers or heterologous DNA probes) and coexpressed. **Table 6** lists selected microorganisms that contain  $\omega$ -cyclic fatty acids.

**Table 6**  
**Examples of microorganisms that contain  $\omega$ -cyclic fatty acids**

Organism	Reference
<i>Curtobacterium pusillum</i>	ATCC19096
<i>Alicyclobacillus acidoterrestris</i>	ATCC49025
<i>Alicyclobacillus acidocaldarius</i>	ATCC27009
<i>Alicyclobacillus cycloheptanicum</i> *	Moore, <i>J. Org. Chem.</i> 62:pp. 2173, 1997.

\*uses cycloheptylcarbonyl-CoA and not cyclohexylcarbonyl-CoA as precursor for cyFA biosynthesis

### C. Ester characteristics

One of ordinary skill in the art will appreciate that an ester includes an A side and a B side. As described herein, the B side is contributed by a fatty acid produced from *de novo* synthesis in the host organism. In some instances where the host is additionally engineered to make alcohols, including fatty alcohols, the A side is also produced by the host organism. In yet other examples the A side can be provided in the medium. As described herein, by selecting the desired thioesterase genes the B side, and when fatty alcohols are being made the A side, can be designed to have certain carbon chain characteristics. These characteristics include points of unsaturation, branching, and desired carbon chain lengths. Exemplary methods of making long chain fatty acid esters, wherein the A and B side are produced by the production host are provided in Example 6, below. Similarly, Example 5 provides methods of making medium chain fatty acid esters. When both the A and B side are contributed by the production host and they are produced using fatty acid biosynthetic pathway intermediates they will have similar carbon chain characteristics. For example, at least 50%, 60%, 70%, or 80% of the fatty acid esters produced will have A sides and B sides that vary by 6, 4, or 2 carbons in length. The A side and the B side will also display similar branching and saturation levels.

In addition to producing fatty alcohols for contribution to the A side, the host can produce other short chain alcohols such as ethanol, propanol, isopropanol, isobutanol, and butanol for incorporation on the A side using techniques well known in the art. For example, butanol can be made by the host organism. To create butanol producing cells, the LS9001 strain (described in Example 1, below) can be further engineered to express *atoB* (acetyl-CoA acetyltransferase) from *Escherichia coli* K12,  $\beta$ -hydroxybutyryl-CoA dehydrogenase from *Butyrivibrio fibrisolvens*, crotonase from *Clostridium beijerinckii*, butyryl CoA dehydrogenase from *Clostridium beijerinckii*, CoA-acylating aldehyde dehydrogenase (ALDH) from *Cladosporium fulvum*, and *adhE* encoding an aldehyde-alcohol dehydrogenase of *Clostridium acetobutylicum* in the pBAD24 expression vector under the *prpBCDE* promoter system. Similarly, ethanol can be produced in a production host using the methods taught by Kalscheuer *et al.*, *Microbiology* 152:2529-2536, 2006, which is herein incorporated by reference.

#### IV. Fermentation

The production and isolation of fatty acid derivatives can be enhanced by employing specific fermentation techniques. One method for maximizing production while reducing costs is increasing the percentage of the carbon source that is converted to hydrocarbon products. During normal cellular lifecycles carbon is used in cellular functions including producing lipids, saccharides, proteins, organic acids, and nucleic acids. Reducing the amount of carbon necessary for growth-related activities can increase the efficiency of carbon source conversion to output. This can be achieved by first growing microorganisms to a desired density, such as a density achieved at the peak of the log phase of growth. At such a point, replication checkpoint genes can be harnessed to stop the growth of cells.

Specifically, quorum sensing mechanisms (reviewed in Camilli and Bassler *Science* 311:1113, 2006; Venturi *FEMS Microbio Rev* 30:274-291, 2006; and Reading and Sperandio *FEMS Microbiol Lett* 254:1-11, 2006) can be used to activate genes such as p53, p21, or other checkpoint genes. Genes that can be activated to stop cell replication and growth in *E. coli* include umuDC genes, the over-expression of which stops the progression from stationary phase to exponential growth (Murli *et al.*, *J. of Bact.* 182:1127, 2000). UmuC is a DNA polymerase that can carry out translesion synthesis over non-coding lesions – the mechanistic basis of most UV and chemical mutagenesis. The *umuDC* gene products are used for the process of translesion synthesis and also serve as a DNA damage checkpoint. UmuDC gene products include UmuC, UmuD, umuD', UmuD'<sub>2</sub>C, UmuD'<sub>2</sub> and UmuD<sub>2</sub>. Simultaneously, the product producing genes would be activated, thus minimizing the need for replication and maintenance pathways to be used while the fatty acid derivative is being made.

The percentage of input carbons converted to hydrocarbon products is a cost driver. The more efficient (i.e. the higher the percentage), the less expensive the process. For oxygen-containing carbon sources (i.e. glucose and other carbohydrate based sources), the oxygen must be released in the form of carbon dioxide. For every 2 oxygen atoms released, a carbon atom is also released leading to a maximal theoretical metabolic efficiency of ~34% (w/w) (for fatty acid derived products).

This figure, however, changes for other hydrocarbon products and carbon sources. Typical efficiencies in the literature are  $\sim < 5\%$ . Engineered microorganisms which produce hydrocarbon products can have greater than 1, 3, 5, 10, 15, 20, 25, and 30% efficiency. In one example microorganisms will exhibit an efficiency of about 10% to about 25%. In other examples, such microorganisms will exhibit an efficiency of about 25% to about 30%, and in other examples such microorganisms will exhibit  $> 30\%$  efficiency.

In some examples where the final product is released from the cell, a continuous process can be employed. In this approach, a reactor with organisms producing fatty acid derivatives can be assembled in multiple ways. In one example, a portion of the media is removed and let to sit. Fatty acid derivatives are separated from the aqueous layer, which will in turn, be returned to the fermentation chamber.

In one example, the fermentation chamber will enclose a fermentation that is undergoing a continuous reduction. In this instance, a stable reductive environment would be created. The electron balance would be maintained by the release of carbon dioxide (in gaseous form). Efforts to augment the NAD/H and NADP/H balance can also facilitate in stabilizing the electron balance.

The availability of intracellular NADPH can be also enhanced by engineering the production host to express an NADH:NADPH transhydrogenase. The expression of one or more NADH:NADPH transhydrogenase converts the NADH produced in glycolysis to NADPH which enhances the production of fatty acid derivatives.

Disclosed herein is a system for continuously producing and exporting fatty acid derivatives out of recombinant host microorganisms via a transport protein. Many transport and efflux proteins serve to excrete a large variety of compounds and can be evolved to be selective for a particular type of fatty acid derivatives. Thus, in some embodiments an exogenous DNA sequence encoding an ABC transporter will be functionally expressed by the recombinant host microorganism, so that the microorganism exports the fatty acid derivative into the culture medium. In one example, the ABC transporter is an ABC transporter from *Caenorhabditis elegans*, *Arabidopsis thaliana*, *Alkaligenes eutrophus* or *Rhodococcus erythropolis* (locus AAN73268). In another example, the ABC transporter is an ABC transporter



chosen from CER5 (locuses At1g51500 or AY734542), AtMRP5, AmiS2 and AtPGP1. In some examples, the ABC transporter is CER5. In yet another example, the CER5 gene is from Arabidopsis (locuses At1g51500, AY734542, At3g21090 and At1g51460).

The transport protein, for example, can also be an efflux protein selected from: AcrAB, TolC and AcrEF from *E. coli*, or tll1618, tll1619 and tll0139 from *Thermosynechococcus elongatus* BP-1.

In addition, the transport protein can be, for example, a fatty acid transport protein (FATP) selected from *Drosophila melanogaster*, *Caenorhabditis elegans*, *Mycobacterium tuberculosis* or *Saccharomyces cerevisiae* or any one of the mammalian FATP's. The FATPs can additionally be resynthesized with the membranous regions reversed in order to invert the direction of substrate flow. Specifically, the sequences of amino acids composing the hydrophilic domains (or membrane domains) of the protein, could be inverted while maintaining the same codons for each particular amino acid. The identification of these regions is well known in the art.

Production hosts can also be chosen for their endogenous ability to release fatty acid derivatives. The efficiency of product production and release into the fermentation broth can be expressed as a ratio intracellular product to extracellular product. In some examples the ratio can be 5:1, 4:1, 3:1, 2:1, 1:1, 1:2, 1:3, 1:4, or 1:5.

The production host can be additionally engineered to express recombinant cellulosomes, such as those described in PCT application number PCT/US2007/003736, which will allow the production host to use cellulosic material as a carbon source. For example, the production host can be additionally engineered to express invertases (EC 3.2.1.26) so that sucrose can be used as a carbon source.

Similarly, the production host can be engineered using the teachings described in U.S. Patent Numbers 5,000,000, 5,028,539, 5,424,202, 5,482,846, and 5,602,030 to Ingram *et al.* so that the production host can assimilate carbon efficiently and use cellulosic materials as carbons sources.

#### **IV. Post production processing**

The fatty acid derivatives produced during fermentation can be separated from the fermentation media. Any technique known for separating fatty acid derivatives from aqueous media can be used. One exemplary separation process provided herein is a two phase (bi-phasic) separation process. This process involves fermenting the genetically engineered production hosts under conditions sufficient to produce a fatty acid derivative, allowing the derivative to collect in an organic phase and separating the organic phase from the aqueous fermentation broth. This method can be practiced in both a batch and continuous fermentation setting.

Bi-phasic separation uses the relative immiscibility of fatty acid derivatives to facilitate separation. Immiscible refers to the relative inability of a compound to dissolve in water and is defined by the compound's partition coefficient. The partition coefficient,  $P$ , is defined as the equilibrium concentration of compound in an organic phase (in a bi-phasic system the organic phase is usually the phase formed by the fatty acid derivative during the production process, however, in some examples an organic phase can be provided (such as a layer of octane to facilitate product separation) divided by the concentration at equilibrium in an aqueous phase (i.e. fermentation broth). When describing a two phase system the  $P$  is usually discussed in terms of  $\log P$ . A compound with a  $\log P$  of 10 would partition 10:1 to the organic phase, while a compound of  $\log P$  of 0.1 would partition 10:1 to the aqueous phase. One of ordinary skill in the art will appreciate that by choosing a fermentation broth and the organic phase such that the fatty acid derivative being produced has a high  $\log P$  value, the fatty acid derivative will separate into the organic phase, even at very low concentrations in the fermentation vessel.

The fatty acid derivatives produced by the methods described herein will be relatively immiscible in the fermentation broth, as well as in the cytoplasm. Therefore, the fatty acid derivative will collect in an organic phase either intracellularly or extracellularly. The collection of the products in an organic phase will lessen the impact of the fatty acid derivative on cellular function and will allow the production host to produce more product. Stated another way, the concentration of the fatty acid derivative will not have as significant of an impact on the host cell.

The fatty alcohols, fatty acid esters, waxes, and hydrocarbons produced as described herein allow for the production of homogeneous compounds wherein at

least 60%, 70%, 80%, 90%, or 95% of the fatty alcohols, fatty acid esters, and waxes produced will have carbon chain lengths that vary by less than 4 carbons, or less than 2 carbons. These compounds can also be produced so that they have a relatively uniform degree of saturation, for example at least 60%, 70%, 80%, 90%, or 95% of the fatty alcohols, fatty acid esters, hydrocarbons and waxes will be mono-, di-, or tri- unsaturated. These compounds can be used directly as fuels, personal care additives, nutritional supplements. These compounds can also be used as feedstock for subsequent reactions for example transesterification, hydrogenation, catalytic cracking via either hydrogenation, pyrolysis, or both or epoxidations reactions to make other products.

## **V. Fuel Compositions**

The fatty acid derivatives described herein can be used as fuel. One of ordinary skill in the art will appreciate that depending upon the intended purpose of the fuel different fatty acid derivatives can be produced and used. For example, for automobile fuel that is intended to be used in cold climates a branched fatty acid derivative may be desirable and using the teachings provided herein, branched hydrocarbons, fatty acid esters, and alcohols can be made. Using the methods described herein fuels comprising relatively homogeneous fatty acid derivatives that have desired fuel qualities can be produced. Such fuels can be characterized by carbon fingerprinting, their lack of impurities when compared to petroleum derived fuels or bio-diesel derived from triglycerides and, moreover, the fatty acid derivative based fuels can be combined with other fuels or fuel additives to produce fuels having desired properties.

### **A. Carbon fingerprinting**

Biologically produced fatty acid derivatives represent a new feedstock for fuels, such as alcohols, diesel and gasoline. Some biofuels made using fatty acid derivatives have not been produced from renewable sources and as such, are new compositions of matter. These new fuels can be distinguished from fuels derived from petrochemical carbon on the basis of dual carbon-isotopic fingerprinting. Additionally, the specific source of biosourced carbon (e.g. glucose vs. glycerol) can

be determined by dual carbon-isotopic fingerprinting (see, US Patent Number 7,169,588, which is herein incorporated by reference).

This method usefully distinguishes chemically-identical materials, and apportions carbon in products by source (and possibly year) of growth of the biospheric (plant) component. The isotopes,  $^{14}\text{C}$  and  $^{13}\text{C}$ , bring complementary information to this problem. The radiocarbon dating isotope ( $^{14}\text{C}$ ), with its nuclear half life of 5730 years, clearly allows one to apportion specimen carbon between fossil ("dead") and biospheric ("alive") feedstocks [Currie, L. A. "Source Apportionment of Atmospheric Particles," Characterization of Environmental Particles, J. Buffle and H. P. van Leeuwen, Eds., 1 of Vol. I of the IUPAC Environmental Analytical Chemistry Series (Lewis Publishers, Inc) (1992) 3 74]. The basic assumption in radiocarbon dating is that the constancy of  $^{14}\text{C}$  concentration in the atmosphere leads to the constancy of  $^{14}\text{C}$  in living organisms. When dealing with an isolated sample, the age of a sample can be deduced approximately by the relationship  $t = (-5730/0.693)\ln(A/A_{\text{sub.O}})$  (Equation 5) where  $t$ =age, 5730 years is the half-life of radiocarbon, and  $A$  and  $A_{\text{sub.O}}$  are the specific  $^{14}\text{C}$  activity of the sample and of the modern standard, respectively [Hsieh, Y., Soil Sci. Soc. Am J., 56, 460, (1992)]. However, because of atmospheric nuclear testing since 1950 and the burning of fossil fuel since 1850,  $^{14}\text{C}$  has acquired a second, geochemical time characteristic. Its concentration in atmospheric  $\text{CO}_2$ --and hence in the living biosphere--approximately doubled at the peak of nuclear testing, in the mid-1960s. It has since been gradually returning to the steady-state cosmogenic (atmospheric) baseline isotope rate ( $^{14}\text{C}/^{12}\text{C}$ ) of ca.  $1.2 \times 10^{-12}$ , with an approximate relaxation "half-life" of 7-10 years. (This latter half-life must not be taken literally; rather, one must use the detailed atmospheric nuclear input/decay function to trace the variation of atmospheric and biospheric  $^{14}\text{C}$  since the onset of the nuclear age.) It is this latter biospheric  $^{14}\text{C}$  time characteristic that holds out the promise of annual dating of recent biospheric carbon.  $^{14}\text{C}$  can be measured by accelerator mass spectrometry (AMS), with results given in units of "fraction of modern carbon" ( $f_M$ ).  $f_M$  is defined by National Institute of Standards and Technology (NIST) Standard Reference Materials (SRMs) 4990B and 4990C, known as oxalic acids standards HOxI and HOxII, respectively. The fundamental definition relates to 0.95 times the

$^{14}\text{C}/^{12}\text{C}$  isotope ratio HOxI (referenced to AD 1950). This is roughly equivalent to decay-corrected pre-Industrial Revolution wood. For the current living biosphere (plant material),  $f_M$  approx 1.1.

The stable carbon isotope ratio ( $^{13}\text{C}/^{12}\text{C}$ ) provides a complementary route to source discrimination and apportionment. The  $^{13}\text{C}/^{12}\text{C}$  ratio in a given biosourced material is a consequence of the  $^{13}\text{C}/^{12}\text{C}$  ratio in atmospheric carbon dioxide at the time the carbon dioxide is fixed and also reflects the precise metabolic pathway. Regional variations also occur. Petroleum, C3 plants (the broadleaf), C.sub.4 plants (the grasses), and marine carbonates all show significant differences in  $^{13}\text{C}/^{12}\text{C}$  and the corresponding delta $^{13}\text{C}$  values. Furthermore, lipid matter of C3 and C4 plants analyze differently than materials derived from the carbohydrate components of the same plants as a consequence of the metabolic pathway. Within the precision of measurement,  $^{13}\text{C}$  shows large variations due to isotopic fractionation effects, the most significant of which for the instant invention is the photosynthetic mechanism. The major cause of differences in the carbon isotope ratio in plants is closely associated with differences in the pathway of photosynthetic carbon metabolism in the plants, particularly the reaction occurring during the primary carboxylation, *i.e.*, the initial fixation of atmospheric  $\text{CO}_2$ . Two large classes of vegetation are those that incorporate the "C3" (or Calvin-Benson) photosynthetic cycle and those that incorporate the "C4" (or Hatch-Slack) photosynthetic cycle. C3 plants, such as hardwoods and conifers, are dominant in the temperate climate zones. In C3 plants, the primary  $\text{CO}_2$  fixation or carboxylation reaction involves the enzyme ribulose-1,5-diphosphate carboxylase and the first stable product is a 3-carbon compound. C4 plants, on the other hand, include such plants as tropical grasses, corn and sugar cane. In C4 plants, an additional carboxylation reaction involving another enzyme, phosphoenol-pyruvate carboxylase, is the primary carboxylation reaction. The first stable carbon compound is a 4-carbon acid which is subsequently decarboxylated. The  $\text{CO}_2$  thus released is refixed by the C3 cycle.

Both C4 and C3 plants exhibit a range of  $^{13}\text{C}/^{12}\text{C}$  isotopic ratios, but typical values are ca. -10 to -14 per mil (C4) and -21 to -26 per mil (C3) [Weber et al., J. Agric. Food Chem., 45, 2942 (1997)]. Coal and petroleum fall generally in this latter range. The  $^{13}\text{C}$  measurement scale was originally defined by a zero set by pee dee

belemnite (PDB) limestone, where values are given in parts per thousand deviations from this material. The " $\Delta^{13}\text{C}$ ", values are in parts per thousand (per mil), abbreviated ‰, and are calculated as follows:

$$\delta^{13}\text{C} \equiv \frac{(^{13}\text{C}/^{12}\text{C})_{\text{sample}} - (^{13}\text{C}/^{12}\text{C})_{\text{standard}}}{(^{13}\text{C}/^{12}\text{C})_{\text{standard}}} \times 100\text{‰} \quad (\text{Equation } 6)$$

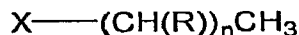
Since the PDB reference material (RM) has been exhausted, a series of alternative RMs have been developed in cooperation with the IAEA, USGS, NIST, and other selected international isotope laboratories. Notations for the per mil deviations from PDB is  $\Delta^{13}\text{C}$ . Measurements are made on  $\text{CO}_2$  by high precision stable ratio mass spectrometry (IRMS) on molecular ions of masses 44, 45 and 46.

The fatty acid derivatives and the associated biofuels, chemicals, and mixtures may be completely distinguished from their petrochemical derived counterparts on the basis of  $^{14}\text{C}$  (fM) and dual carbon-isotopic fingerprinting, indicating new compositions of matter.

The fatty acid derivatives described herein have utility in the production of biofuels and chemicals. The new fatty acid derivative based product compositions provided by the instant invention additionally may be distinguished on the basis of dual carbon-isotopic fingerprinting from those materials derived solely from petrochemical sources. The ability to distinguish these products is beneficial in tracking these materials in commerce. For example, fuels or chemicals comprising both "new" and "old" carbon isotope profiles may be distinguished from fuels and chemicals made only of "old" materials. Hence, the instant materials may be followed in commerce on the basis of their unique profile and for the purposes of defining competition, and for determining shelf life.

In some examples a biofuel composition is made that includes a fatty acid derivative having  $\delta^{13}\text{C}$  of from about -10.9 to about -15.4, wherein the fatty acid derivative accounts for at least about 85% of biosourced material (derived from a renewable resource such as cellulosic materials and sugars) in the composition. In

other examples, the biofuel composition includes a fatty acid derivative having the formula



wherein X represents  $\text{CH}_3$ ,  $-\text{CH}_2\text{OR}^1$ ;  $-\text{C}(\text{O})\text{OR}^2$ ; or  $-\text{C}(\text{O})\text{NR}^3\text{R}^4$ ;

R is, for each n, independently absent, H or lower aliphatic;

n is an integer from 8 to 34, such as from 10 to 24; and

$\text{R}^1$ ,  $\text{R}^2$ ,  $\text{R}^3$  and  $\text{R}^4$  independently are selected from H and lower alkyl.

Typically, when R is lower aliphatic, R represents a branched, unbranched or cyclic lower alkyl or lower alkenyl moiety. Exemplary R groups include, without limitation, methyl, isopropyl, isobutyl, *sec*-butyl, cyclopentenyl and the like. The fatty acid derivative is additionally characterized as having a  $\delta^{13}\text{C}$  of from about -10.9 to about -15.4; and the fatty acid derivative accounts for at least about 85% of biosourced material in the composition. In some examples the fatty acid derivative in the biofuel composition is characterized by having a fraction of modern carbon ( $f_M^{14}\text{C}$ ) of at least about 1.003, 1.010, or 1.5.

#### **B. Fatty acid derivatives**

The centane number (CN), viscosity, melting point, and heat of combustion for various fatty acid esters have been characterized in for example, Knothe, *Fuel Processing Technology* 86:1059-1070, 2005, which is herein incorporated by reference. Using the teachings provided herein a production host can be engineered to produce anyone of the fatty acid esters described in the Knothe, *Fuel Processing Technology* 86:1059-1070, 2005.

Alcohols (short chain, long chain, branched or unsaturated) can be produced by the production hosts described herein. Such alcohols can be used as fuels directly or they can be used to create an ester, i.e. the A side of an ester as described above. Such ester alone or in combination with the other fatty acid derivatives described herein are useful a fuels.

Similarly, hydrocarbons produced from the microorganisms described herein can be used as biofuels. Such hydrocarbon based fuels can be designed to contain branch points, defined degrees of saturation, and specific carbon lengths. When

used as biofuels alone or in combination with other fatty acid derivatives the hydrocarbons can be additionally combined with additives or other traditional fuels (alcohols, diesel derived from triglycerides, and petroleum based fuels).

### **C. Impurities**

The fatty acid derivatives described herein are useful for making bio-fuels. These fatty acid derivatives are made directly from fatty acids and not from the chemical processing of triglycerides. Accordingly, fuels comprising the disclosed fatty acid derivatives will contain less of the impurities than are normally associated with bio-fuels derived from triglycerides, such as fuels derived from vegetable oils and fats.

The crude fatty acid derivative bio-fuels described herein (prior to mixing the fatty acid derivative with other fuels such as traditional fuels) will contain less transesterification catalyst than petrochemical diesel or bio-diesel. For example, the fatty acid derivative can contain less than about 2%, 1.5%, 1.0%, 0.5%, 0.3%, 0.1%, 0.05%, or 0% of a transesterification catalyst or an impurity resulting from a transesterification catalyst. Transesterification catalysts include for example, hydroxide catalysts such as NaOH, KOH, LiOH, and acidic catalysts, such as mineral acid catalysts and Lewis acid catalysts. Catalysts and impurities resulting from transesterification catalysts include, without limitation, tin, lead, mercury, cadmium, zinc, titanium, zirconium, hafnium, boron, aluminum, phosphorus, arsenic, antimony, bismuth, calcium, magnesium, strontium, uranium, potassium, sodium, lithium, and combinations thereof.

Similarly, the crude fatty acid derivative bio-fuels described herein (prior to mixing the fatty acid derivative with other fuels such as petrochemical diesel or bio-diesel) will contain less glycerol (or glycerin) than bio-fuels made from triglycerides. For example, the fatty acid derivative can contain less than about 2%, 1.5%, 1.0%, .5%, .3%, .1%, .05%, or 0% glycerol.

The crude biofuel derived from fatty acid derivatives will also contain less free alcohol (i.e. alcohol that is used to create the ester) than bio-diesel made from triglycerides. This is in-part due to the efficiency of utilization of the alcohol by the production host. For example, the fatty acid derivative will contain less than about 2%, 1.5%, 1.0%, .5%, .3%, .1%, .05%, or 0% free alcohol.



Biofuel derived from the disclosed fatty acid derivatives can be additionally characterized by its low concentration of sulfur compared to petroleum derived diesel. For example, biofuel derived from fatty acid derivatives can have less than about 2%, 1.5%, 1.0%, .5%, .3%, .1%, .05%, or 0% sulfur.

#### **D. Additives**

Fuel additives are used to enhance the performance of a fuel or engine. For example, fuel additives can be used to alter the freezing/gelling point, cloud point, lubricity, viscosity, oxidative stability, ignition quality, octane level, and flash point. In the United States, all fuel additives must be registered with Environmental Protection Agency and companies that sell the fuel additive and the name of the fuel additive are publicly available on the agency website and also by contacting the agency. One of ordinary skill in the art will appreciate that the fatty acid derivatives described herein can be mixed with one or more such additives to impart a desired quality.

One of ordinary skill in the art will also appreciate that the fatty acid derivatives described herein are can be mixed with other fuels such as bio-diesel derived from triglycerides, various alcohols such as ethanol and butanol, and petroleum derived products such as gasoline. In some examples, a fatty acid derivative, such as C16:1 ethyl ester or C18:1 ethyl ester, is produced which has a low gel point. This low gel point fatty acid derivative is mixed with bio-diesel made from triglycerides to lessen the overall gelling point of the fuel. Similarly, a fatty acid derivative such as C16:1 ethyl ester or C18:1 ethyl ester can be mixed with petroleum derived diesel to provide a mixture that is at least and often greater than 5% biodiesel. In some examples, the mixture includes at least 20% or greater of the fatty acid derivative.

For example, a biofuel composition can be made that includes at least about 20%, 30%, 40%, 50%, 60%, 70%, 80%, 85%, 90% or 95% of a fatty acid derivative that includes a carbon chain that is 8:0, 10:0, 12:0, 14:0, 14:1, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3, 20:0, 20:1, 20:2, 20:3, 22:0, 22:1 or 22:3. Such biofuel compositions can additionally include at least one additive selected from a cloud point lowering additive that can lower the cloud point to less than about 5°C, or 0°C, a surfactant, or a microemulsion, at least about 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70% or

80%, 85%, 90%, or 95% diesel fuel from triglycerides; petroleum derived gasoline or diesel fuel from petroleum.

## EXAMPLES

**Fig. 1** is a diagram of the FAS pathway showing the enzymes directly involved in the synthesis of acyl-ACP. To increase the production of waxes/fatty acid esters, and fatty alcohols one or more of the enzymes can be over expressed or mutated to reduce feedback inhibition. Additionally, enzymes that metabolize the intermediates to make non-fatty acid based products (side reactions) can be functionally deleted or attenuated to increase the flux of carbon through the fatty acid biosynthetic pathway. Examples 1, 2, and 8 below provide exemplary production hosts that have been modified to increase fatty acid production.

**Figs. 2, 3 and 4** show biosynthetic pathways that can be engineered to make fatty alcohols and wax/fatty acid esters, respectively. As illustrated in **Fig. 2** the conversion of each substrate (acetyl-CoA, malonyl-CoA, acyl-ACP, fatty acid, and acyl-CoA) to each product (acetyl-CoA, malonyl-CoA, acyl-ACP, fatty acid, and acyl-CoA) can be accomplished using several different polypeptides that are members of the enzyme classes indicated. The Examples below describe microorganisms that have been engineered or can be engineered to produce specific fatty alcohols and waxes/fatty acid esters and hydrocarbons.

### Example 1, Production Host Construction

An exemplary production host is LS9001. LS9001 was produced by modifying C41(DE3) from Overexpress.com (Saint Beausine, France) to functionally deleting the *fadE* gene (acyl-CoA dehydrogenase).

Briefly, the *fadE* knock-out strain of *E. coli* was made using primers YafV\_NotI and Ivry\_OI to amplify about 830 bp upstream of *fadE* and primers Lpcaf\_ol and LpcaR\_Bam to amplify about 960 bp downstream of *fadE*. Overlap PCR was used to create a construct for in frame deletion of the complete *fadE* gene. The *fadE* deletion construct was cloned into the temperature sensitive plasmid pKOV3, which contained a *SacB* gene for counterselection, and a chromosomal deletion of *fadE* was made according to the method of Link *et al.*, *J. Bact.* 179:6228-

6237, 1997. The resulting strain was not capable of degrading fatty acids and fatty acyl-CoAs (this functional deletion is herein designated as  $\Delta$ *fadE*)

Additional modifications that can be included in a production host include introducing a plasmid carrying the four genes which are responsible for acetyl-CoA carboxylase activity in *E. coli* (*accA*, *B*, *C*, and *D*, Accessions: NP\_414727, NP\_417721, NP\_417722, NP\_416819, EC 6.4.1.2). The *accABCD* genes were cloned in two steps as bicistronic operons into the *NcoI/HindIII* and *NdeI/AvrII* sites of pACYCDuet-1 (Novagen, Madison, WI) the resulting plasmid was termed pAS004.126.

Additional modifications that can be included in a production host include the following: over-expression of *aceEF* (encoding the E1p dehydrogase component and the E2p dihydrolipoamide acyltransferase component of the pyruvate and 2-oxoglutarate dehydrogenase complexes); and *fabH/fabD/fabG/acpP/fabF* (encoding FAS) from any organism known in the art to encode such proteins, including for example *E. coli*, *Nitrosomonas europaea* (ATCC 19718), *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Streptomyces spp*, *Ralstonia*, *Rhodococcus*, *Corynebacteria*, *Brevibacteria*, *Mycobacteria*, oleaginous yeast, and the like can be expressed in the production host. Similarly, production hosts can be engineered to express *accABCD* (encoding acetyl co-A carboxylase) from *Pisum sativum* instead of, or in addition to, the *E.coli* homologues. However, when the production host is also producing butanol it is less desirable to express the *Pisum sativum* homologue.

In some exemplary production hosts, genes can be knocked out or attenuated using the method of Link, *et al.*, *J. Bacteriol.* 179:6228-6237, 1997. For example, genes that can be knocked out or attenuated include *gpsA* (encoding biosynthetic sn-glycerol 3-phosphate dehydrogenase, accession NP\_418065, EC: 1.1.1.94); *ldhA* (encoding lactate dehydrogenase, accession NP\_415898, EC: 1.1.1.28); *pflb* (encoding formate acetyltransferase 1, accessions: P09373, EC: 2.3.1.54); *adhE* (encoding alcohol dehydrogenase, accessions: CAA47743, EC: 1.1.1.1, 1.2.1.10); *pta* (encoding phosphotransacetylase, accessions: NP\_416800, EC: 2.3.1.8); *poxB* (encoding pyruvate oxidase, accessions: NP\_415392, EC: 1.2.2.2); *ackA* (encoding acetate kinase, accessions: NP\_416799, EC: 2.7.2.1) and combinations thereof.

Similarly, the PlsB[D311E] mutation can be introduced into LS9001 to attenuate PlsB using the method described above for the *fadE* deletion. Once introduced, this mutation will decrease the amount of carbon being diverted to phospholipid production (see, Fig. 1). Briefly, an allele encoding PlsB[D311E] is made by replacing the GAC codon for aspartate 311 with a GAA codon for glutamate. The altered allele is made by gene synthesis and the chromosomal *plsB* wildtype allele is exchanged for the mutant *plsB*[D311E] allele using the method of Link *et al.* (see above).

## Example 2, Production host modifications

The following plasmids were constructed for the expression of various proteins that are used in the synthesis of fatty acid derivatives. The constructs were made using standard molecular biology methods and all the cloned genes were put under the control of IPTG-inducible promoters (T7, tac or lac promoters).

The '*tesA* gene (thioesterase A gene accession NP\_415027 without leader sequence (Cho and Cronan, *The J. of Biol. Chem.*, 270:4216-9, 1995, EC: 3.1.1.5, 3.1.2.-) of *E. coli* was cloned into *NdeI*/*AvrII* digested pETDuet-1 (pETDuet-1 described herein is available from Novagen, Madison, WI). Genes encoding for FatB-type plant thioesterases (TEs) from *Umbellularia California*, *Cuphea hookeriana* and *Cinnamomum camphorum* (accessions: UcFatB1=AAA34215, ChFatB2=AAC49269, ChFatB3=AAC72881, CcFatB=AAC49151 were individually cloned into three different vectors: (i) *NdeI*/*AvrII* digested pETDuet-1, (ii) *XhoI*/*HindIII* digested pBluescript KS+ (Stratagene, La Jolla, CA)(used to create N-terminal lacZ::TE fusion proteins) and (iii) *XbaI*/*HindIII* digested pMAL-c2X (New England Lab, Ipswich, MA) (used to create n-terminal MalE::TE fusions). The *fadD* gene (encoding acyl-CoA synthetase) from *E. coli* was cloned into a *NcoI*/*HindIII* digested pCDFDuet-1 derivative, which contained the *acrI* gene (acyl-CoA reductase) from *Acinetobacter baylyi* ADP1 within its *NdeI*/*AvrII* sites. Table 7 provides a summary of the plasmids generated to make several exemplary production strains, one of ordinary skill in the art will appreciate that different plasmids and genomic modifications can be used to achieve similar strains.

**Table 7**  
**Summary of Plasmids used in Production hosts**

Plasmid	Source Organism Gene Product	Accession No., EC number
pETDuet-1- <i>tesA</i>	<i>E. coli</i> TesA	Accessions: NP_415027, EC: 3.1.1.5, 3.1.2.-
pETDuet-1-TEuc pBluescript-TEuc pMAL-c2X-TEuc	<i>Umbellularia</i> <i>California</i> UcFatB1	Q41635 AAA34215
pETDuet-1-TEch pBluescript-TEch pMAL-c2X-TEch	<i>Cuphea hookeriana</i> ChFatB2 ChFatB3	ABB71581 AAC49269 AAC72881
pETDuet-1-TEcc pBluescript-TEcc TEci	<i>Cinnamomum</i> <i>camphorum</i> CcFatB	AAC49151
pCDFDuet-1- fadD-acr1	<i>E. coli</i>	fadD:Accessions NP_416319, EC 6.2.1.3 acr1:Accessions YP_047869

The chosen expression plasmids contain compatible replicons and antibiotic resistance markers, so that a four-plasmid expression system can be established. Therefore, LS9001 can be co-transformed with (i) any of the TE-expressing plasmids, (ii) the FadD-expressing plasmid, which also expresses *acr1* and (iii) wax synthase expression plasmid. When induced with IPTG, the resulting strain will produce increased concentrations of fatty-alcohols from carbon sources such as glucose. The carbon chain length and degree of saturation of the fatty alcohol produced is dependent on the thioesterase gene that is expressed.

### **Example 3, Production of fatty alcohol in the recombinant *E. coli* strain**

Fatty alcohols were produced by expressing a thioesterase gene and an acyl-CoA reductase gene (FAR) exogenously in a production host. More specifically, plasmids pCDFDuet-1-fadD-acr1 (acyl-CoA reductase) and pETDuet-1-*tesA* (thioesterase) were transformed into *E. coli* strain LS9001 (described in Example 1) and corresponding transformants were selected in LB plate supplemented with 100 mg/L of spectinomycin and 50 mg/L of carbenicillin. Four transformants of

LS9001/pCDFDuet-1-fadD-acr1 were independently inoculated into 3 mL of M9 medium supplemented with 50 mg/L of carbenicillin and 100 mg/L of spectinomycin). The samples containing the transformants were grown in at 25°C in a shaker (250 rpm) until they reached 0.5 OD<sub>600</sub>. 1.5 mL of each sample was transferred into a 250 mL flask containing 30 mL of the medium described above. The resulting culture was grown at 25°C in a shaker until the culture reached between 0.5 -1.0 OD<sub>600</sub>. IPTG was then added to a final concentration of 1 mM. and growth continued for 40 hours.

The cells were then spun down at 4000 rpm and the cell pellets were suspended in 1.0 mL of methanol. 3 mL of ethyl acetate was then mixed with the suspended cells. 3 mL of H<sub>2</sub>O were then added to the mixture and the mixture was sonicated for 20 minutes. The resulting sample was centrifuged at 4000 rpm for 5 minutes and the organic phase (the upper phase) which contained fatty alcohol and was subjected to GC/MS analysis. Total alcohol (including tetradecanol, hexadecanol, hexadecenol and octadecenol) yield was about 1-10 mg/L. When an *E. coli* strain carrying only empty vectors was cultured in the same way, only 0.2-0.5 mg/L of fatty alcohols were found in the ethyl acetate extract.

#### **Example 4, Production and release of fatty alcohol from production host**

Acr1 (acyl-CoA reductase) was expressed in *E. coli* grown on glucose as the sole carbon and energy source. The *E. coli* produced small amounts of fatty alcohols such as dodecanol (C12:0-OH), tetradecanol (C14:0-OH) and hexadecanol (C16:0-OH). In other samples, *FadD* (acyl-CoA synthetase) was expressed together with *acr1* in *E. coli* and a five-fold increase in fatty alcohol production was observed.

In other samples, *acr1*, *fadD*, *accABCD* (acetyl-CoA Carboxylase) (plasmid carrying *accABCD* constructed as described in Example 1) were expressed along with various individual thioesterases (TEs) in wildtype *E. coli* C41(DE3) and an *E. coli* C41(DE3  $\Delta$ *fadE*), a strain lacking acyl-CoA dehydrogenase. This resulted in additional increases in fatty alcohol production and modulating the profiles of fatty alcohols (see Fig. 5). For example, over-expression of *E. coli* 'tesA (pETDuet-1-'tesA) in this system achieved approximately a 60-fold increase in C12:0-OH,

C14:0-OH and C16:0-OH with C14:0-OH being the major fatty alcohol. A very similar result was obtained when the ChFatB3 enzyme (FatB3 from *Cuphea hookeriana* in pMAL-c2X-TEcu) was expressed. When the UcFatB1 enzyme (FatB1 from *Umbellularia californica* in pMAL-c2X-TEuc) was expressed, fatty alcohol production increased approximately 20-fold and C12:0-OH was the predominant fatty alcohol.

Expression of ChFatB3 and UcFatB1 also led to the production of significant amounts of the unsaturated fatty alcohols C16:1-OH and C14:1-OH, respectively. The presence of fatty alcohols was also found in the supernatant of samples generated from the expression of *tesA* (Fig. 6). At 37°C approximately equal amounts of fatty alcohols were found in the supernatant and in the cell pellet, whereas at 25°C approximately 25% of the fatty alcohols were found in the supernatant.

#### **Example 5, Medium Chain fatty acid esters**

Alcohol acetyl transferases (AATs, EC 2.3.1.84), which is responsible for acyl acetate production in various plants, can be used to produce medium chain length waxes, such as octyl octanoate, decyl octanoate, decyl decanoate, and the like. Fatty esters, synthesized from medium chain alcohol (such as C6, C8) and medium chain acyl-CoA (or fatty acids, such as C6 or C8) have a relative low melting point. For example, hexyl hexanoate has a melting point of -55°C and octyl octanoate has a melting point of -18 to -17°C. The low melting points of these compounds makes them good candidates for use as biofuels.

In this example, a SAAT gene was co-expressed in a production host C41(DE3,  $\Delta$ fadE) with *fadD* from *E. coli* and *acr1* (alcohol reductase from *A. baylyi* ADP1) and octanoic acid was provided in the fermentation broth. This resulted in the production of octyl octanoate. Similarly, when the wax synthase gene from *A. baylyi* ADP1 was expressed in the production host instead of the SAAT gene octyl octanoate was produced.

A recombinant SAAT gene was synthesized using DNA 2.0 (Menlo Park, CA 94025). The synthesized DNA was based on the published gene sequence (accession number AF193789) and modified to eliminate the *NcoI* site. The

synthesized *SAAT* gene (as a *Bam*HI-*Hind*III fragment) was cloned in pRSET B (Invitrogen, Calsbad, California), linearized with *Bam*HI and *Hind*III. The resulted plasmid, pHZ1.63A was cotransformed into an *E. coli* production host with pAS004.114B, which carries a *fadD* gene from *E. coli* and *acr1* gene from *A. baylyi* ADP1. The transformants were grown in 3 mL of M9 medium with 2% of glucose. After IPTG induction and the addition of 0.02% of octanoic acid, the culture was continued at 25°C from 40 hours. After that, 3 mL of acetyl acetate was added to the whole culture and mixed several times with mixer. The acetyl acetate phase was analyzed by GC/MS.

Surprising, in the acetyl acetate extract, there is no acyl acetate found. However, a new compound was found and the compound was octyl octanoate. Whereas the control strain without the *SAAT* gene [C41(DE3,  $\Delta$ *fadE*)/pRSET B+pAS004.114B] did not produce octyl octanoate. Also the strain [C41(DE3,  $\Delta$ *fadE*)/pHZ1.43 B+pAS004.114B], in which the wax synthase gene from *A. baylyi* ADP1 was carried by pHZ1.43 produced octyl octanoate (see Figs. 7B).

The finding that *SAAT* activity produces octyl octanoate has not reported before and makes it possible to produce medium chain waxes such as octyl octanoate, octyl decanoate, which have low melting point and are good candidates to be use for biofuel to replace triglyceride based biodiesel.

#### **Example 6, Production of wax ester in *E. coli* strain LS9001**

Wax esters were produced by engineering an *E. coli* production host to express a fatty alcohol forming acyl-CoA reductase, thioesterase, and a wax synthase. Thus, the production host produced both the A and the B side of the ester and the structure of both sides was influenced by the expression of the thioesterase gene.

More specifically, wax synthase from *A. baylyi* ADP1 (termed WSadp1, accessions AA017391, EC: 2.3.175) was amplified with the following primers using genomic DNA from *A. baylyi* ADP1 as the template. The primers were (1) WSadp1\_*Nde*I, 5'-TCATATGCGCCCATTACATCCG-3' and (2) WSadp1\_*Avr*, 5'- TCCTAGGAGGGCTAATTTAGCCCTTTAGTT-3'. The PCR product was digested with *Nde*I and *Avr*II and cloned into pCOALDeut-1 to give pHZ 1.43. The



plasmid carrying WSadp1 was then co-transformed into *E. coli* strain LS9001 with both pETDuet-1'tesA and pCDFDuet-1-fadD-acr1 and transformants were selected in LB plates supplemented with 50 mg/L of kanamycin, 50 mg/L of carbenicillin and 100 mg/L of spectinomycin. Three transformants were inoculated in 3 mL of LBKCS (LB broth supplement with 50 mg/L of kanamycin, 50 mg/L of carbenicillin, 100 mg/L of spectinomycin and 10 g/L of glucose) and cultured at 37°C shaker (250 rpm). When the cultures reached 0.5 OD<sub>600</sub>, 1.5 mL of each culture was transferred into 250 mL flasks containing 50 mL of LBKCS and the flasks were grown in a shaker (250 rpm) at 37°C until the culture reached 0.5-1.0 OD<sub>600</sub>. IPTG was then added to a final concentration of 1 mM. The induced cultures were grown at 37°C shaker for another 40-48 hours.

The culture was then placed into 50 mL conical tubes and the cells were spun down at 3500 X g for 10 minutes. The cell pellet was then mixed with 5 mL of ethyl acetate. The ethyl acetate extract was analyzed with GC/MS. The intracellular yield of waxes (including C16C16, C14:1C16, C18:1C18:1, C2C14, C2C16, C2C16:1, C16C16:1 and C2C18:1) was about 10 mg/L. When an *E. coli* strain only carrying empty vectors was cultured in the same way, only 0.2 mg/L of wax was found in the ethyl acetate extract.

#### **Example 7, Production and release of fatty -ethyl ester from production host**

The LS9001 strain was modified by transforming it with the plasmids carrying a wax synthase gene from *A. baylyi* (plasmid pHZ1.43), a thioesterase gene from *Cuphea hookeriana* (plasmid pMAL-c2X-TEcu) and a *fadD* gene from *E. coli* (plasmid pCDFDuet-1-fadD). This recombinant strain was grown at 25°C in 3 mL of M9 medium with 50mg/L of kanamycin, 100 mg/L of carbenicillin and 100 mg/L of spectinomycin. After IPTG induction, the media was adjusted to a final concentration of 1% ethanol and 2% glucose. The culture was allowed to grow for 40 hours after IPTG induction. The cells were separated from the spent medium by centrifugation at 3500 X g for 10 minutes). The cell pellet was re-suspended with 3 mL of M9 medium. The cell suspension and the spent medium were then extracted with 1 volume of ethyl acetate. The resulting ethyl acetate phases from the cells suspension and the supernatant were subjected to GC-MS analysis. The results

showed that the C16 ethyl ester was the most prominent ester species (as expected for this thioesterase, see Table 1), and that 20% of the fatty acid ester produced was released from the cell (see Fig. 8). A control *E. coli* strain C41(DE3,  $\Delta$ fadE) containing pCOLADuet-1 (empty vector for the wax synthase gene), pMAL-c2X-TEuc (containing *fatB* from *U. californica*) and pCDFDuet-1-fadD (*fadD* gene from *E. coli*) failed to produce detectable amounts of fatty ethyl esters. The fatty acid esters were quantified using commercial palmitic acid ethyl ester as the reference. Fatty acid esters were also made using the methods described herein except that methanol, or isopropanol was added to the fermentation broth and the expected fatty acid esters were produced.

**Example 8, The influence of various thioesterases on the composition of fatty-ethyl esters produced in recombinant *E. coli* strains.**

The thioesterases FatB3 (*C. hookeriana*), TesA (*E. coli*), and FatB (*U. californica*) were expressed simultaneously with wax synthase (*A. baylyi*). A plasmid termed pHZ1.61 was constructed by replacing the *NotI*-*AvrII* fragment (carrying the *acrI* gene) with the *NotI*-*AvrII* fragment from pHZ1.43 so that *fadD* and the *ADP1* wax synthase were in one plasmid and both coding sequences were under the control of separate T7 promoter. The construction of pHZ1.61 made it possible to use a two plasmid system instead of the three plasmid system as described in Example 6. pHZ1.61 was then co-transformed into *E. coli* C41(DE3,  $\Delta$ fadE) with one of the various plasmids carrying the different thioesterase genes stated above.

The total fatty acid ethyl esters (supernatant and intracellular fatty acid ethyl esters) produced by these transformants were evaluated using the technique described herein. The yields and the composition of fatty acid ethyl esters are summarized in Table 8.

**Table 8**

**The yields (mg/L) and the composition of fatty acid ethyl esters by recombinant *E. coli* C41(DE3,  $\Delta$ fadE)/pHZ1.61 and plasmids carrying various thioesterase genes.**

Thioesterases	C2C10	C2C12:1	C2C12	C2C14:1	C2C14	C2C16:1	C2C16	C2C18:1	Total
'TesA	0.0	0.0	6.5	0.0	17.5	6.9	21.6	18.1	70.5
ChFatB3	0.0	0.0	0.0	0.0	10.8	12.5	11.7	13.8	48.8
ucFatB	6.4	8.5	25.3	14.7	0.0	4.5	3.7	6.7	69.8
pMAL	0.0	0.0	0.0	0.0	5.6	0.0	12.8	7.6	26.0

Note: 'TesA, pETDuet-1-'*tesA*; chFatB3, pMAL-c2X-TEcu; ucFatB, pMAL-c2X-TEuc; pMAL, pMAL-c2X, the empty vector for thioesterase genes used in the study.

### **Example 9, Production Host Construction**

The genes that control fatty acid production are conserved between microorganisms. For example, **Table 9** identifies the homologues of many of the genes described herein which are known to be expressed in microorganisms that produce hydrocarbons. To increase fatty acid production and, therefore, hydrocarbon production in microorganisms such as those identified in **Table 9**, heterologous genes, such as those from *E. coli* can be expressed. One of ordinary skill in the art will also appreciate that genes that are endogenous to the microorganisms provided in **Table 9** can also be over-expressed, or attenuated using the methods described herein. Moreover, genes that are described in **Fig. 10** can be expressed or attenuated in microorganisms that endogenously produce hydrocarbons to allow for the production of specific hydrocarbons with defined carbon chain length, saturation points, and branch points.

For example, exogenous nucleic acid sequences encoding acetyl-CoA carboxylase are introduced into *K. radiotolerans*. The following genes comprise the acetyl-CoA carboxylase protein product in *K. radiotolerans*; acetyl CoA carboxylase, alpha subunit (*accA*/ ZP\_00618306), acetyl-CoA carboxylase, biotin carboxyl carrier protein (*accB*/ ZP\_00618387), acetyl-CoA carboxylase, biotin carboxylase subunit (*accC* / ZP\_00618040), and acetyl-CoA carboxylase, beta (carboxyltransferase) subunit (*accD*/ ZP\_00618306). These genes are cloned into a plasmid such that they make a synthetic acetyl-CoA carboxylase operon (*accABCD*)

under the control of a *K. radiotolerans* expression system such as the expression system disclosed in Ruyter *et al.*, *Appl Environ Microbiol.* 62:3662-3667, 1996. Transformation of the plasmid into *K. radiotolerans* will enhance fatty acid production. The hydrocarbon producing strain of *K. radiotolerans* can also be engineered to make branched, unsaturated hydrocarbons having specific carbon chain lengths using the methods disclosed herein.

**Table 9**  
**Hydrocarbon production hosts**

<u>Organism</u>	<u>Gene Name</u>	<u>Accession No./Seq ID/Loci</u>	<u>EC No.</u>
<i>Desulfovibrio desulfuricans</i> G20	accA	YP_388034	6.4.1.2
<i>Desulfovibrio desulfuricans</i> G22	accC	YP_388573/YP_388033	6.3.4.14, 6.4.1.2
<i>Desulfovibrio desulfuricans</i> G23	accD	YP_388034	6.4.1.2
<i>Desulfovibrio desulfuricans</i> G28	fabH	YP_388920	2.3.1.180
<i>Desulfovibrio desulfuricans</i> G29	fabD	YP_388786	2.3.1.39
<i>Desulfovibrio desulfuricans</i> G30	fabG	YP_388921	1.1.1.100
			3.1.26.3,
<i>Desulfovibrio desulfuricans</i> G31	acpP	YP_388922/YP_389150	1.6.5.3, 1.6.99.3
<i>Desulfovibrio desulfuricans</i> G32	fabF	YP_388923	2.3.1.179
<i>Desulfovibrio desulfuricans</i> G33	gpsA	YP_389667	1.1.1.94
			1.1.1.27,
<i>Desulfovibrio desulfuricans</i> G34	ldhA	YP_388173/YP_390177	1.1.1.28
<i>Erwinia (micrococcus) amylovora</i>	accA	942060 - 943016	6.4.1.2
<i>Erwinia (micrococcus) amylovora</i>	accB	3440869 - 3441336	6.4.1.2
<i>Erwinia (micrococcus) amylovora</i>	accC	3441351 - 3442697	6.3.4.14, 6.4.1.2
<i>Erwinia (micrococcus) amylovora</i>	accD	2517571 - 2516696	6.4.1.2
<i>Erwinia (micrococcus) amylovora</i>	fadE	1003232 - 1000791	1.3.99.-
<i>Erwinia (micrococcus) amylovora</i>	plsB(D311E)	333843 - 331423	2.3.1.15
<i>Erwinia (micrococcus) amylovora</i>	aceE	840558 - 843218	1.2.4.1
<i>Erwinia (micrococcus) amylovora</i>	aceF	843248 - 844828	2.3.1.12
<i>Erwinia (micrococcus) amylovora</i>	fabH	1579839 - 1580789	2.3.1.180
<i>Erwinia (micrococcus) amylovora</i>	fabD	1580826 - 1581749	2.3.1.39
<i>Erwinia (micrococcus) amylovora</i>	fabG	CAA74944	1.1.1.100
			3.1.26.3,
<i>Erwinia (micrococcus) amylovora</i>	acpP	1582658 - 1582891	1.6.5.3, 1.6.99.3
<i>Erwinia (micrococcus) amylovora</i>	fabF	1582983 - 1584221	2.3.1.179
<i>Erwinia (micrococcus) amylovora</i>	gpsA	124800 - 125810	1.1.1.94
			1.1.1.27,
<i>Erwinia (micrococcus) amylovora</i>	ldhA	1956806 - 1957789	1.1.1.28
<i>Kineococcus radiotolerans</i> SRS30216	accA	ZP_00618306	6.4.1.2
<i>Kineococcus radiotolerans</i> SRS30216	accB	ZP_00618387	6.4.1.2
<i>Kineococcus radiotolerans</i> SRS30216	accC	ZP_00618040 /ZP_00618387	6.3.4.14, 6.4.1.2

<i>Kineococcus radiotolerans</i> SRS30216	accD	ZP_00618306	6.4.1.2
<i>Kineococcus radiotolerans</i> SRS30216	fadE	ZP_00617773	1.3.99.-
<i>Kineococcus radiotolerans</i> SRS30216	plsB(D311E)	ZP_00617279	2.3.1.15
<i>Kineococcus radiotolerans</i> SRS30216	aceE	ZP_00617600	1.2.4.1
<i>Kineococcus radiotolerans</i> SRS30216	aceF	ZP_00619307	2.3.1.12
<i>Kineococcus radiotolerans</i> SRS30216	fabH	ZP_00618003	2.3.1.180
<i>Kineococcus radiotolerans</i> SRS30216	fabD	ZP_00617602	2.3.1.39
<i>Kineococcus radiotolerans</i> SRS30216	fabG	ZP_00615651	1.1.1.100 3.1.26.3,
<i>Kineococcus radiotolerans</i> SRS30216	acpP	ZP_00617604	1.6.5.3, 1.6.99.3
<i>Kineococcus radiotolerans</i> SRS30216	fabF	ZP_00617605	2.3.1.179
<i>Kineococcus radiotolerans</i> SRS30216	gpsA	ZP_00618825	1.1.1.94 1.1.1.27,
<i>Kineococcus radiotolerans</i> SRS30216	ldhA	ZP_00618879	1.1.1.28
<i>Rhodospirillum rubrum</i>	accA	YP_425310	6.4.1.2
<i>Rhodospirillum rubrum</i>	accB	YP_427521	6.4.1.2
		YP_427522/YP_425144/YP_427028/YP_426209/	
<i>Rhodospirillum rubrum</i>	accC	YP_427404	6.3.4.14, 6.4.1.2
<i>Rhodospirillum rubrum</i>	accD	YP_428511	6.4.1.2
<i>Rhodospirillum rubrum</i>	fadE	YP_427035	1.3.99.-
<i>Rhodospirillum rubrum</i>	aceE	YP_427492	1.2.4.1
<i>Rhodospirillum rubrum</i>	aceF	YP_426966	2.3.1.12
<i>Rhodospirillum rubrum</i>	fabH	YP_426754	2.3.1.180
<i>Rhodospirillum rubrum</i>	fabD	YP_425507	2.3.1.39
<i>Rhodospirillum rubrum</i>	fabG	YP_425508/YP_425365	1.1.1.100 3.1.26.3,
			1.6.5.3, 1.6.99.3
<i>Rhodospirillum rubrum</i>	acpP	YP_425509	
		YP_425510/YP_425510	
<i>Rhodospirillum rubrum</i>	fabF	/YP_425285	2.3.1.179
<i>Rhodospirillum rubrum</i>	gpsA	YP_428652	1.1.1.94 1.1.1.27,
			1.1.1.28
<i>Rhodospirillum rubrum</i>	ldhA	YP_426902/YP_428871	
<i>Vibrio furnissii</i>	accA		1, 16 6.4.1.2
<i>Vibrio furnissii</i>	accB		2, 17 6.4.1.2
<i>Vibrio furnissii</i>	accC		3, 18 6.3.4.14, 6.4.1.2
<i>Vibrio furnissii</i>	accD		4, 19 6.4.1.2
<i>Vibrio furnissii</i>	fadE		5, 20 1.3.99.-
<i>Vibrio furnissii</i>	plsB(D311E)		6, 21 2.3.1.15
<i>Vibrio furnissii</i>	aceE		7, 22 1.2.4.1
<i>Vibrio furnissii</i>	aceF		8, 23 2.3.1.12
<i>Vibrio furnissii</i>	fabH		9, 24 2.3.1.180
<i>Vibrio furnissii</i>	fabD		10, 25 2.3.1.39

<i>Vibrio furnissii</i>	fabG		11, 26	1.1.1.100 3.1.26.3,
<i>Vibrio furnissii</i>	acpP		12, 27	1.6.5.3, 1.6.99.3
<i>Vibrio furnissii</i>	fabF		13, 28	2.3.1.179
<i>Vibrio furnissii</i>	gpsA		14, 29	1.1.1.94 1.1.1.27,
<i>Vibrio furnissii</i>	ldhA		15, 30	1.1.1.28
<i>Stenotrophomonas maltophilia</i> R551-3	accA	ZP_01643799		6.4.1.2
<i>Stenotrophomonas maltophilia</i> R551-3	accB	ZP_01644036		6.4.1.2
<i>Stenotrophomonas maltophilia</i> R551-3	accC	ZP_01644037		6.3.4.14, 6.4.1.2
<i>Stenotrophomonas maltophilia</i> R551-3	accD	ZP_01644801		6.4.1.2
<i>Stenotrophomonas maltophilia</i> R551-3	fadE	ZP_01645823		1.3.99.-
<i>Stenotrophomonas maltophilia</i> R551-3	plsB(D311E)	ZP_01644152		2.3.1.15
<i>Stenotrophomonas maltophilia</i> R551-3	aceE	ZP_01644724		1.2.4.1
<i>Stenotrophomonas maltophilia</i> R551-3	aceF	ZP_01645795		2.3.1.12
<i>Stenotrophomonas maltophilia</i> R551-3	fabH	ZP_01643247		2.3.1.180
<i>Stenotrophomonas maltophilia</i> R551-3	fabD	ZP_01643535		2.3.1.39
<i>Stenotrophomonas maltophilia</i> R551-3	fabG	ZP_01643062		1.1.1.100 3.1.26.3,
<i>Stenotrophomonas maltophilia</i> R551-3	acpP	ZP_01643063		1.6.5.3, 1.6.99.3
<i>Stenotrophomonas maltophilia</i> R551-3	fabF	ZP_01643064		2.3.1.179
<i>Stenotrophomonas maltophilia</i> R551-3	gpsA	ZP_01643216		1.1.1.94 1.1.1.27,
<i>Stenotrophomonas maltophilia</i> R551-3	ldhA	ZP_01645395		1.1.1.28

For Table 9, Accession Numbers are from GenBank, Release 159.0 as of April 15 2007,

EC Numbers are from KEGG, Release 42.0 as of April 2007 (plus daily updates up to and including 05/09/07), results for *Erwinia amylovora* strain Ea273 are taken from the Sanger sequencing center, completed shotgun sequence as of 5/9/07, positions for *Erwinia* represent locations on the Sanger psuedo-chromosome, sequences from *Vibrio furnisii* M1 are from the LS9 VFMI pseudochromosome, v2 build, as of 9/28/06, and include the entire gene, and may also include flanking sequence

#### Example 10, Additional Exemplary Production strains

Table 10, below provides additional exemplary production strains. Two example biosynthetic pathways are described for producing fatty acids, fatty

alcohols, and wax esters. A genetically engineered host can be produced by cloning the expression of the accABCD genes from *E. coli*, the 'tesA gene from *E. coli*, and *fadD* gene from *E. coli* into a host cell. Host cells can be selected from *E. coli*, yeast, add to the list. These genes can also be transformed into a host cell that is modified to contain one or more of the genetic manipulations described in Examples 1 and 2, above. As provided in Table 10, additional production hosts can be created using the indicated exogenous genes.

**Table 10**  
**Combination of genes useful for making genetically engineered production strains**

Peptide	Sources of genes	Genes	Fatty acids		Fatty alcohols		wax /fatty esters	
			example 1	example 2	example 1	example 2	example 1	example 2
acetyl-CoA carboxylase	<i>E. coli</i>	accABCD	X	X	X	X	X	X
thio-esterase	<i>E. coli</i>	tesA	X		X		X	X
	<i>Cinnamomum camphora</i>	ccFatB						
	<i>Umbellularia californica</i>	umFatB		X		X		
	<i>Cuphea hookeriana</i>	chFatB2						
	<i>Cuphea hookeriana</i>	chFatB3						
	<i>Cuphea hookerian</i>	chFatA						
	<i>Arabidopsis thaliana</i>	AtFatA1						
	<i>Arabidopsis thalian</i>	AtFatB1[M141T]						
acyl-CoA synthase	<i>E.coli</i>	fadD	X	X	X	X	X	X
acyl-CoA reductase	<i>Bombyx mori</i>	bFAR						
	<i>Acinetobacter baylyi</i> ADP1	acr 1			X		X	
	<i>Simmondsia chinensis</i>	jjFAR				X		X
	<i>Triticum aestivum</i>							

	<i>Mus musculus</i>	mFAR1						
	<i>Mus musculus</i>	mFAR2						
	<i>Acinetobacter</i> sp M1	acr M1						
	<i>Homo sapiens</i>	hFAR						
wax synthase /alcohol acyltransferase	<i>Fundibacter jadensis</i> DSM 12178	WST9						
	<i>Acinetobacter</i> sp. HO1-N	WSHN					x	
	<i>Acinetobacter baylyi</i> ADP1	WSadp1						x
	<i>Mus musculus</i>	mWS						
	<i>Homo sapiens</i>	hWS						
	<i>Fragaria x ananassa</i>	SAAT						
	<i>Malus x domestica</i>	MpAAT						
	<i>Simmondsia chinensis</i>	JjWS (AAD38041)						
Decarbonylase	<i>Arabidopsis thaliana</i>	cer1						
	<i>Oryza sativa</i>	cer1						
Transporter	<i>Acinetobacter</i> sp. HO1-N						x	x
	<i>Arabidopsis thaliana</i>	Cer5						

### Example 11, Fermentation

Host microorganisms can be also engineered to express umuC and umuD from *E. coli* in pBAD24 under the *prpBCDE* promoter system through *de novo* synthesis of this gene with the appropriate end-product production genes. For small scale hydrocarbon product production, *E. coli* BL21(DE3) cells harbouring pBAD24 (with ampicillin resistance and the end-product synthesis pathway) as well as pUMVC1 (with kanamycin resistance and the acetyl CoA/malonyl CoA over-expression system) are incubated overnight at at 37°C shaken at >200 rpm 2L flasks in 500 ml LB medium supplemented with 75 µg/mL ampicillin and 50 µg/ml



kanamycin until cultures reached an OD<sub>600</sub> of > 0.8. Upon achieving an OD<sub>600</sub> of > 0.8, cells are supplemented with 25 mM sodium propionate (pH 8.0) to activate the engineered gene systems for production as well as to stop cellular proliferation (through activation of umuC and umuD proteins). Induction is performed for 6 hours at 30°C. After incubation, media is examined for product using GC-MS (as described below).

For large scale product production, the engineered microorganisms are grown in 10 L, 100 L or larger batches, fermented and induced to express desired products based on the specific genes encoded in plasmids as appropriate. *E. coli* BL21(DE3) cells harbouring pBAD24 (with ampicillin resistance and the end-product synthesis pathway) as well as pUMVC1 (with kanamycin resistance and the acetyl-CoA/malonyl-CoA over-expression system) are incubated from a 500 mL seed culture for 10 L fermentations (5 L for 100 L fermentations) in LB media (glycerol free) at 37°C shaken at >200 rpm until cultures reached an OD<sub>600</sub> of > 0.8 (typically 16 hours) incubated with 50 µg/mL kanamycin and 75 µg/mL ampicillin. Media is treated with continuously supplemented to maintain a 25 mM sodium propionate (pH 8.0) to activate the engineered in gene systems for production as well as to stop cellular proliferation (through activation of umuC and umuD proteins). Media is continuously supplemented with glucose to maintain a concentration 90g/100 mL. After the first hour of induction, aliquots of no more than 10% of the total cell volume are removed each hour and allowed to sit unagitated so as to allow the hydrocarbon product to rise to the surface and undergo a spontaneous phase separation. The hydrocarbon component is then collected and the aqueous phase returned to the reaction chamber. The reaction chamber is operated continuously. When the OD<sub>600</sub> drops below 0.6, the cells are replaced with a new batch grown from a seed culture.

For wax ester production, subsequent to isolation, the wax esters are washed briefly in 1 M HCl to split the ester bond, and returned to pH 7 with extensive washing with distilled water.

#### **Example 12, Product Characterization**

To characterize and quantify the fatty alcohols and fatty acid esters, gas chromatography (GC) coupled with electron impact mass spectra (MS) detection was used. Fatty alcohol samples were first derivatized with an excess of N-trimethylsilyl (TMS) imidazole to increase detection sensitivity. Fatty acid esters did not required derivatization. Both fatty alcohol-TMS derivatives and fatty acid esters were dissolved in an appropriate volatile solvent, like ethyl acetate. The samples were analyzed on a 30m DP-5 capillary column using the following method. After a 1  $\mu$ L splitless injection onto the GC/MS column, the oven is held at 100°C for 3 minutes. The temperature was ramped up to 320°C at a rate of 20°C/minute. The oven was held at 320°C for an additional 5 minutes. The flow rate of the carrier gas helium was 1.3 mL/minute. The MS quadrapole scans from 50 to 550 m/z. Retention times and fragmentation patterns of product peaks were compared with authentic references to confirm peak identity.

For example, hexadeconic acid ethyl ester eluted at 10.18 minutes (**Figs. 9A and 9B**). The parent ion of 284 mass units was readily observed. More abundant were the daughter ions produced during mass fragmentation. This included the most prevalent daughter ion of 80 mass units. The derivatized fatty alcohol hexadecanol-TMS eluted at 10.29 minutes and the parent ion of 313 could be observed. The most prevalent ion was the M-14 ion of 299 mass units.

Quantification was carried out by injecting various concentrations of the appropriate authentic references using the GC/MS method described above. This information was used to generate a standard curve with response (total integrated ion count) versus concentration.

### **EQUIVALENTS**

While specific examples of the subject inventions are explicitly disclosed herein, the above specification and examples herein are illustrative and not restrictive. Many variations of the inventions will become apparent to those skilled in the art upon review of this specification including the examples. The full scope of the inventions should be determined by reference to the examples, along with their full scope of equivalents, and the specification, along with such variations.

What is claimed is:

1. A microorganism comprising one or more exogenous nucleic acid sequences encoding at least one peptide selected from accA (EC 6.4.1.2), accB (EC 6.4.1.2), accC (EC 6.4.1.2), accD (EC 6.4.1.2), aceE (EC 1.2.4.1, 2.3.1.61, 2.3.1.12), aceF (EC 1.2.4.1, 2.3.4.16, 2.3.1.12), acpP (AAC74178), fadD (EC 2.3.1.86), cer1 (EC 4.1.99.5), fabA (EC 4.2.1.60), fabB (EC 2.3.1.41), fabD (EC 2.3.1.39), fabG (EC 1.1.1.100), fabH (EC 2.3.1.180), fabI (EC 1.3.1.9), fabZ (EC 4.2.1.-), lipase (EC 3.1.1.3), malonyl-CoA decarboxylase (EC 4.1.1.9, 4.1.1.41), panD (EC 4.1.1.11), panK (EC 2.7.1.33), pdh (EC 1.2.4.1), udhA (EC 1.6.1.1) and combinations thereof, and a peptide comprising a wax synthase (EC 2.3.1.75).
2. A microorganism comprising one or more exogenous nucleic acid sequences encoding at least one peptide selected from accA (EC 6.4.1.2), accB (EC 6.4.1.2), accC (EC 6.4.1.2), accD (EC 6.4.1.2), aceE (EC 1.2.4.1, 2.3.1.61, 2.3.1.12), aceF (EC 1.2.4.1, 2.3.4.16, 2.3.1.12), acpP (AAC74178), fadD (EC 2.3.1.86), cer1 (EC 4.1.99.5), fabA (EC 4.2.1.60), fabB (EC 2.3.1.41), fabD (EC 2.3.1.39), fabG (EC 1.1.1.100), fabH (EC 2.3.1.180), fabI (EC 1.3.1.9), fabZ (EC 4.2.1.-), lipase (EC 3.1.1.3), malonyl-CoA decarboxylase (EC 4.1.1.9, 4.1.1.41), panD (EC 4.1.1.11), panK (EC 2.7.1.33), pdh (EC 1.2.4.1), udhA (EC 1.6.1.1) and combinations thereof, and a peptide comprising an alcohol acetyltransferase (2.3.1.84).
3. A microorganism comprising one or more exogenous nucleic acid sequences encoding at least one peptide selected from accA (EC 6.4.1.2), accB (EC 6.4.1.2), accC (EC 6.4.1.2), accD (EC 6.4.1.2), aceE (EC 1.2.4.1, 2.3.1.61, 2.3.1.12), aceF (EC 1.2.4.1, 2.3.4.16, 2.3.1.12), acpP (AAC74178), fadD (EC 2.3.1.86), cer1 (EC 4.1.99.5), fabA (EC 4.2.1.60), fabB (EC 2.3.1.41), fabD (EC 2.3.1.39), fabG (EC 1.1.1.100), fabH (EC 2.3.1.180), fabI (EC 1.3.1.9), fabZ (EC 4.2.1.-), lipase (EC 3.1.1.3), malonyl-CoA decarboxylase (EC 4.1.1.9, 4.1.1.41), panD (EC 4.1.1.11), panK (EC 2.7.1.33), pdh (EC 1.2.4.1), udhA (EC 1.6.1.1) combinations thereof, and a peptide comprising an alcohol dehydrogenase (EC 1.1.1.1).

4. A microorganism comprising one or more exogenous nucleic acid sequences encoding at least one peptide selected from *accA* (EC 6.4.1.2), *accB* (EC 6.4.1.2), *accC* (EC 6.4.1.2), *accD* (EC 6.4.1.2), *aceE* (EC 1.2.4.1, 2.3.1.61, 2.3.1.12), *aceF* (EC 1.2.4.1, 2.3.4.16, 2.3.1.12), *acpP* (AAC74178), *fadD* (EC 2.3.1.86), *cer1* (EC 4.1.99.5), *fabA* (EC 4.2.1.60), *fabB* (EC 2.3.1.41), *fabD* (EC 2.3.1.39), *fabG* (EC 1.1.1.100), *fabH* (EC 2.3.1.180), *fabI* (EC 1.3.1.9), *fabZ* (EC 4.2.1.-), *lipase* (EC 3.1.1.3), *malonyl-CoA decarboxylase* (EC 4.1.1.9, 4.1.1.41), *panD* (EC 4.1.1.11), *panK* (EC 2.7.1.33), *pdh* (EC 1.2.4.1), *udhA* (EC 1.6.1.1) and combinations thereof, and a peptide comprising a fatty alcohol forming acyl-CoA reductase (1.1.1.\*).

5. A microorganism comprising one or more attenuated endogenous nucleic acid sequences selected from *ackA* (EC 2.7.2.1), *ackB* (EC 2.7.2.1), *adhE* (EC 1.1.1.1, 1.2.1.10), *fabF* (EC 2.3.1.179), *fabR* (accession NP\_418398), *fadE* (EC 1.3.99.3, 1.3.99.-), *GST* (EC 6.3.2.3), *gpsA* (EC 1.1.1.94), *ldhA* (EC 1.1.1.28), *pflB* (EC 2.3.1.54), *plsB* (EC 2.3.1.15), *poxB* (EC 1.2.2.2), *pta* (EC 2.3.1.8), *glutathione synthase* (EC 6.3.2.3) and combinations thereof, and one or more exogenous nucleic acid sequences encoding a second peptide comprising a wax synthase (EC 2.3.1.75) or an acyltransferase (EC 2.3.1.84).

6. A microorganism comprising one or more attenuated endogenous nucleic acid sequences selected from *ackA* (EC 2.7.2.1), *ackB* (EC 2.7.2.1), *adhE* (EC 1.1.1.1, 1.2.1.10), *fabF* (EC 2.3.1.179), *fabR* (accession NP\_418398), *fadE* (EC 1.3.99.3, 1.3.99.-), *GST* (EC 6.3.2.3), *gpsA* (EC 1.1.1.94), *ldhA* (EC 1.1.1.28), *pflB* (EC 2.3.1.54), *plsB* (EC 2.3.1.15), *poxB* (EC 1.2.2.2), *pta* (EC 2.3.1.8), *glutathione synthase* (EC 6.3.2.3) and combinations thereof, and one or more exogenous nucleic acid sequences encoding a peptide comprising an alcohol acetyltransferase (EC 2.3.1.84).

7. A microorganism comprising one or more attenuated endogenous nucleic acid sequences selected from *ackA* (EC 2.7.2.1), *ackB* (EC 2.7.2.1), *adhE* (EC 1.1.1.1, 1.2.1.10), *fabF* (EC 2.3.1.179), *fabR* (accession NP\_418398), *fadE* (EC

1.3.99.3, 1.3.99.-), GST (EC 6.3.2.3), *gpsA* (EC 1.1.1.94), *ldhA* (EC 1.1.1.28), *pflB* (EC 2.3.1.54), *plsB* (EC 2.3.1.15), *poxB* (EC 1.2.2.2), *pta* (EC 2.3.1.8), glutathione synthase (EC 6.3.2.3) and combinations thereof, and one or more exogenous nucleic acid sequences encoding a peptide comprising an alcohol dehydrogenase (EC 1.1.1.1).

8. A microorganism comprising one or more attenuated endogenous nucleic acid sequences selected from *ackA* (EC 2.7.2.1), *ackB* (EC 2.7.2.1), *adhE* (EC 1.1.1.1, 1.2.1.10), *fabF* (EC 2.3.1.179), *fabR* (accession NP\_418398), *fadE* (EC 1.3.99.3, 1.3.99.-), GST (EC 6.3.2.3), *gpsA* (EC 1.1.1.94), *ldhA* (EC 1.1.1.28), *pflB* (EC 2.3.1.54), *plsB* (EC 2.3.1.15), *poxB* (EC 1.2.2.2), *pta* (EC 2.3.1.8), glutathione synthase (EC 6.3.2.3) and combinations thereof, and one or more exogenous nucleic acid sequences encoding a peptide comprising a fatty alcohol forming acyl-CoA reductase (1.2.1.\*).

9. The microorganism of any one of claims 1-8, wherein the microorganism is an *E. coli*.

10. The microorganism of any one of claims 1-8, wherein the microorganism additionally comprises a fatty acid derivative.

11. The microorganism of claim 10, wherein the microorganism additionally comprises an exogenous nucleic sequence encoding ACP, Sfa, or combinations thereof.

12. The microorganism of claim 10, wherein the microorganism expresses one or more exogenous nucleic acid sequences encoding an enzyme selected from one or more components of the branch chain keto acid dehydrogenase complex (EC 1.2.4.4), *llve* (EC 2.6.1.42), *lpd* (EC 1.8.1.4), *Ccr* (EC1.1.19), *IcmA* (EC5.4.99.2), *IcmB* (5.4.99.13), *fabH* (EC 2.3.1.180), *fabF* (EC 2.3.1.179), *fabH3* (EC 2.3.1.180), *fabC3*(NP\_823468), beta-ketoacyl-ACP synthase II (EC 2.3.1.180), enoyl-CoA

reductase (EC 1.3.1.34), enoyl-CoA isomerase (EC 4.2.1.-), and combinations thereof, wherein the fatty acid derivative is branched.

13. The microorganism of claim 10, wherein the microorganism expresses one or more exogenous nucleic acid sequences encoding a thioesterase (3.1.2.-, 3.1.1.-).

14. The microorganism of claim 10, wherein the microorganism expresses one or more exogenous nucleic acid sequences encoding an enzyme selected from *fabB* (EC2.3.1.41), *fabK* (EC 1.2.1.9), *fabL* (EC 1.2.1.9), *fabM* (5.3.3.14), *fadE* (EC 1.3.99.3, 1.3.99.-) and combinations thereof and wherein the fatty acid derivative is unsaturated.

15. The microorganism of any one of claims 1-8, wherein *fadE* is attenuated.

16. The microorganism of any one of claims 1-8, wherein *accABCE*, *fadD* are over-expressed.

17. The microorganism of any one of claims 1-8, wherein the microorganism is in a vessel comprising a fermentation broth comprising at least 10 mg/L fatty acid ester, 10 mg/L fatty alcohol, 10 mg/L hydrocarbon or at least 10 mg/L wax.

18. The microorganism of claim 10, wherein the fatty acid derivative comprises from about 1 to about 5 double bonds.

19. The microorganism of claim 10, wherein the fatty acid derivative comprises a carbon chain length of from about 8 to about 30.

20. The microorganism of claim 10, wherein the fatty acid derivative comprises from about 1 to about 5 branch points.

21. The microorganism of any one of claims 1-8, wherein the microorganism additionally comprises a fatty acid ester or wax having an A side and B side.

22. The microorganism of claim 21, wherein the A side and the B side are produced by the microorganism.

23. The microorganism of claim 21, wherein the A side, B side, or both the A side and the B side, comprise from about 1 to about 5 double bonds.

24. The microorganism of claim 21, wherein the A side, B side, or both the A side and the B side, comprise a carbon chain length of from about 1 to about 26.

25. The microorganism of claim 21, wherein the A side, B side, or both the A side and the B side, comprise from about 1 to about 5 carbon branch points.

26. The microorganism of claim 21, wherein the A side, B side, or both the A side and the B side, comprise between 1 and 5 cyclopropyl moieties.

27. The microorganism of any one of claims 1-8, wherein the microorganism is *Arthrobacter sp.*, *Bacillus sp.*, *Botryococcus braunii*, *Chromatium sp.*, *Cladosporium resina* (ATCC22711), *Clostridium pasteurianum* VKM, *Clostridium tenanomorphum*, *Clostridium acidurici*, *Corynebacterium species*, *cyanobacterial species* (*Nostoc muscorum*, *Anacystis* (*Synechococcus*) *nidulans*, *Phormidium luridum*, *Chlorogloea fritschii*, *Trichodesmium erythaeum*, *Oscillatoria williamsii*, *Microcoleus chthonoplaseis*, *Coccochloris elabens*, *Agmenellum quadruplicatum*, *Plectonema terebrans*, *M. vaginatus*, and *C. scopulorum*) , *Desulfovibrio desulfuricans* (ATCC29577), *Kineococcus radiotolerans* (BAA-149), *Micrococcus luteus* (FD533, ATCC 272, 381, 382, ISU, 540, 4698, 7468, 27141), *Micrococcus sp.* (ATCC 146, 398, 401, 533), *Micrococcus roseus* (ATCC 412, 416, 516), *Micrococcus lysodeikticus*, *Mycobacterium species*, *Penicillium sp.*, *Aspergillus sp.*, *Trichoderma virida*, *Pullularia pullulans*, *Jeotgalicoccus sp.* (*M. candidans*)(ATCC 8456), *Rhodopseudomonas spheroids* *Chlorobium sp.*, *Rhodospirillum rubrum* (ATCC11170), *Rhodomicrobium vannielii*, *Stenotrophomonas maltophilia* (ATCC 13637, 17444, 17445, 17666, 17668, 17673, 17674, 17679, 17677), *Saccharomycodes ludwigii* (ATCC 22711), *Saccharomyces sp.* (*oviformus*, *ludwigii*,

*tropicalis*), *Vibrio furnissii* M1, *Vibrio marinus* MP-1, *Vibrio ponticus*, *Serratia marinorubra*, *Ustilago maydis*, *Ustilago nuda*, *Urocystis agropyri*, *Sphacelotheca reiliana*, or *Tilletia* sp. (*foetida*, *caries*, *controversa*).

28. A method of producing a fatty alcohol comprising culturing the microorganism of any one of claims 3, 4, 7 or 8 under conditions sufficient to produce a fatty alcohol; and separating the fatty alcohol.

29. A method of producing a fatty acid ester comprising culturing the microorganism of any one of claims 1, 2, 6, 7 or 21-25 under conditions sufficient to produce a fatty acid ester; and separating the fatty acid ester.

30. A method of producing a wax comprising culturing the microorganism of any one of claims 1, 2, 6, 7 or 21-25 under conditions sufficient to produce a wax; and separating the wax.

31. A microorganism selected from *Arthrobacter* sp., *Bacillus* sp., *Botryococcus braunii*, *Chromatium* sp., *Cladosporium resina* (ATCC22711), *Clostridium pasteurianum* VKM, *Clostridium tenanomorphum*, *Clostridium acidurici*, *Corynebacterium* species, cyanobacterial species (*Nostoc muscorum*, *Anacystis* (*Synechococcus*) *nidulans*, *Phormidium luridum*, *Chlorogloea fritschii*, *Trichodesmium erythaeum*, *Oscillatoria williamsii*, *Microcoleus chthonoplaseis*, *Coccochloris elabens*, *Agmenellum quadruplicatum*, *Plectonema terebrans*, *M. vaginatus*, and *C. scopulorum*), *Desulfovibrio desulfuricans* (ATCC29577), *Kineococcus radiotolerans* (BAA-149), *Micrococcus luteus* (FD533, ATCC 272, 381, 382, ISU, 540, 4698, 7468, 27141), *Micrococcus* sp. (ATCC 146, 398, 401, 533), *Micrococcus roseus* (ATCC 412, 416, 516), *Micrococcus lysodeikticus*, *Mycobacterium* species, *Penicillium* sp., *Aspergillus* sp., *Trichoderma virida*, *Pullularia pullulans*, *Jeotgalicoccus* sp. (*M. candicans*) (ATCC 8456), *Rhodopseudomonas spheroids* *Chlorobium* sp., *Rhodospirillum rubrum* (ATCC11170), *Rhodomicrobium vannielii*, *Stenotrophomonas maltophilia* (ATCC



13637, 17444, 17445, 17666, 17668, 17673, 17674, 17679, 17677), *Saccharomycodes ludwigii* (ATCC 22711), *Saccharomyces sp.* (*oviformis*, *ludwigii*, *tropicalis*), *Vibrio furnissii* M1, *Vibrio marinus* MP-1, *Vibrio ponticus*, *Serratia marinorubra*, *Ustilago maydis*, *Ustilago nuda*, *Urocystis agropyri*, *Sphacelotheca reiliana*, or *Tilletia sp.* (*foetida*, *caries*, *controversa*) comprising one or more exogenous nucleic acid sequences encoding a first polypeptide selected from *accA* (EC 6.4.1.2), *accB* (EC 6.4.1.2), *accC* (EC 6.4.1.2), *accD* (EC 6.4.1.2), *aceE* (EC 1.2.4.1, 2.3.1.61, 2.3.1.12), *aceF* (EC 1.2.4.1, 2.3.4.16, 2.3.1.12), *acpP* (AAC74178), *fadD* (EC 2.3.1.86), *cer1* (EC 4.1.99.5), *fabA* (EC 4.2.1.60), *fabB* (EC 2.3.1.41), *fabD* (EC 2.3.1.39), *fabG* (EC 1.1.1.100), *fabH* (EC 2.3.1.180), *fabI* (EC 1.3.1.9), *fabZ* (EC 4.2.1.-), *lipase* (EC 3.1.1.3), *malonyl-CoA decarboxylase* (EC 4.1.1.9, 4.1.1.41), *panD* (EC 4.1.1.11), *panK* (EC 2.7.1.33), *pdh* (EC 1.2.4.1), *udhA* (EC 1.6.1.1) and combinations thereof, wherein the microorganism produces increased amounts of hydrocarbons compared to the wild-type microorganism.

32. A microorganism selected from *Arthrobacter sp.*, *Bacillus sp.*, *Botryococcus braunii*, *Chromatium sp.*, *Cladosporium resina* (ATCC22711), *Clostridium pasteurianum* VKM, *Clostridium tenanomorphum*, *Clostridium acidurici*, *Corynebacterium species*, *cyanobacterial species* (*Nostoc muscorum*, *Anacystis* (*Synechococcus*) *nidulans*, *Phormidium luridum*, *Chlorogloea fritschii*, *Trichodesmium erythraeum*, *Oscillatoria williamsii*, *Microcoleus chthonoplaste*s, *Coccochloris elabens*, *Agmenellum quadruplicatum*, *Plectonema terebrans*, *M. vaginatus*, and *C. scopulorum*), *Desulfovibrio desulfuricans* (ATCC29577), *Kineococcus radiotolerans* (BAA-149), *Micrococcus luteus* (FD533, ATCC 272, 381, 382, ISU, 540, 4698, 7468, 27141), *Micrococcus sp.* (ATCC 146, 398, 401, 533), *Micrococcus roseus* (ATCC 412, 416, 516), *Micrococcus lysodeikticus*, *Mycobacterium species*, *Penicillium sp.*, *Aspergillus sp.*, *Trichoderma virida*, *Pullularia pullulans*, *Jeotgalicoccus sp.* (*M. candicans*) (ATCC 8456), *Rhodopseudomonas spheroids* *Chlorobium sp.*, *Rhodospirillum rubrum* (ATCC11170), *Rhodomicrobium vannielii*, *Stenotrophomonas maltophilia* (ATCC 13637, 17444, 17445, 17666, 17668, 17673, 17674, 17679, 17677), *Saccharomycodes ludwigii* (ATCC 22711), *Saccharomyces sp.* (*oviformis*, *ludwigii*,

*tropicalis*), *Vibrio furnissii* M1, *Vibrio marinus* MP-1, *Vibrio ponticus*, *Serratia marinorubra*, *Ustilago maydis*, *Ustilago nuda*, *Urocystis agropyri*, *Sphacelotheca reiliana*, and *Tilletia* sp. (*foetida*, *caries*, *controversa*) comprising one or more attenuated endogenous nucleic acid sequences selected from *ackA* (EC 2.7.2.1), *ackB* (EC 2.7.2.1), *adhE* (EC 1.1.1.1, 1.2.1.10), *fabF* (EC 2.3.1.179), *fabR* (accession NP\_418398), *fadE* (EC 1.3.99.3, 1.3.99.-), GST (EC 6.3.2.3), *gpsA* (EC 1.1.1.94), *ldhA* (EC 1.1.1.28), *pflB* (EC 2.3.1.54), *plsB* (EC 2.3.1.15), *poxB* (EC 1.2.2.2), *pta* (EC 2.3.1.8), glutathione synthase (EC 6.3.2.3) and combinations thereof, wherein the microorganism produces increased amounts of hydrocarbons compared to the wild-type microorganism.

33. The microorganism of any one of claims 31 or 32, wherein the microorganism additionally expresses an enzyme selected from a wax synthase (EC 2.3.1.75), an alcohol acetyltransferase (2.3.1.84), an alcohol dehydrogenase (EC 1.1.1.1), and a fatty alcohol forming acyl-CoA reductase (1.1.1.\*).

34. The microorganism of any one of claims 31 and 32, wherein the microorganism expresses one or more nucleic acid sequences encoding an enzyme selected from one or more components of the branch chain keto acid dehydrogenase complex (EC 1.2.4.4), *Ilve* (EC 2.6.1.42), *lpd* (EC 1.8.1.4), *ccr* (EC1.1.19), *IcmA* (EC5.4.99.2), *IcmB* (5.4.99.13), *fabH* (EC 2.3.1.180), ACP (accession NP\_626635), *fabF* (EC 2.3.1.179), *fabH3* (EC 2.3.1.180), *fabC3* (NP\_823468), beta-ketoacyl-ACP synthase II (EC 2.3.1.180), enoyl-CoA reductase (EC 1.3.1.34), enoyl-CoA isomerase (EC 4.2.1.-), and combinations thereof, wherein the fatty acid derivative is branched.

35. The microorganism of any one of claims 31 and 32, wherein the microorganism expresses one or more exogenous nucleic acid sequences encoding a thioesterase (3.1.2.-, 3.1.1.-).

36. The microorganism of any one of claims 31 and 32, wherein the microorganism expresses one or more exogenous nucleic acid sequences encoding

an enzyme selected from FadA (EC 2.3.1.16), FadI (EC 2.3.1.16), FadB (EC 2.3.1.41), FadJ (EC 4.2.1.17, EC 5.1.2.3, EC 5.3.3.8, EC 1.1.1.35), FabK (EC 1.2.1.9), FabL (EC 1.2.1.9), FabM (5.3.3.14) and combinations thereof.

37. A method of obtaining a purified fatty acid derivative comprising, culturing a microorganism of any one of claims 1-28, and 31-36, under conditions sufficient to produce a fatty acid derivative; allowing the fatty acid derivative to separate into an organic phase; and purifying the fatty acid derivative from the organic phase.

38. A biofuel composition, comprising:  
at least about 85% of a fatty acid derivative, wherein the fatty acid derivative comprises a carbon chain selected from the group consisting of 8:0, 10:0, 12:0, 14:0, 14:1, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3, 20:0, 20:1, 20:2, 20:3, 22:0, 22:1 or 22:3;  
and  
at least one additive sufficient to lower the cloud point of the biofuel composition to less than about 0 °C.

39. A biofuel composition, comprising:  
at least about 17% of a fatty acid derivative, wherein the fatty acid derivative comprises a carbon chain selected from the group consisting of 8:0, 10:0, 12:0, 14:0, 14:1, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3, 20:0, 20:1, 20:2, 20:3, 22:0, 22:1 or 22:3;  
and  
at least about 80% conventional diesel fuel.

40. A biofuel composition or feedstock, comprising a fatty acid derivative having  $\delta^{13}\text{C}$  of from about -10.9 to about -15.4, wherein the fatty acid derivative accounts for at least about 85% of biosourced material in the composition.

41. A biofuel composition or feedstock, comprising a fatty acid derivative of the formula



wherein X represents  $CH_3$ ,  $-CH_2OR^1$ ;  $-C(O)OR^2$ ; or  $-C(O)NR^3R^4$ ;

R is, for each n, independently absent, H or lower aliphatic;

n is an integer from 8 to 34; and

$R^1$ ,  $R^2$ ,  $R^3$  and  $R^4$  independently are selected from H and lower alkyl;

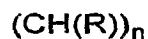
wherein the fatty acid derivative has a  $\delta^{13}C$  of from about -10.9 to about -15.4; and the fatty acid derivative accounts for at least about 85% of biosourced material in the composition.

42. The biofuel composition of claim 40, wherein the fatty acid derivative accounts for at least about 85% of biosourced fatty acid-derived material in the composition.

43. The biofuel composition or feedstock of claim 41, wherein the fatty acid derivative has a fraction of modern carbon ( $f_M^{14}C$ ) of at least about 1.003.

44. The biofuel composition or feedstock of claim 41, wherein R is, for each n, independently selected from H, methyl, ethyl, isopropyl, isobutyl, sec-butyl and cyclopentenyl.

45. The biofuel composition or feedstock of claim 41, wherein the formula

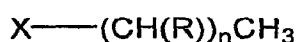


comprises at least one alkenyl moiety.

46. The biofuel composition or feedstock of claim 41, wherein the fatty acid derivative comprises a carbon chain selected from the group consisting of 8:0, 10:0, 12:0, 14:0, 14:1, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3, 20:0, 20:1, 20:2, 20:3, 22:0, 22:1 or 22:3.

47. The biofuel composition or feedstock of claim 41, further comprising a lower alcohol in the biofuel composition.

48. The biofuel composition of claim 47, wherein the lower alcohol is selected from ethanol, butanol, hexanol or combinations thereof.
49. The biofuel composition of claim 47, further comprising a surfactant.
50. The biofuel composition of claim 47, wherein the biofuel composition comprises a microemulsion.
51. A biofuel composition, comprising:  
     at least about 55% of a fatty acid derivative wherein the fatty acid derivative comprises a carbon chain selected from the group consisting of 8:0, 10:0, 12:0, 14:0, 14:1, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3, 20:0, 20:1, 20:2, 20:3, 22:0, 22:1 or 22:3;  
     and  
     at least one additive sufficient to lower the cloud point of the biofuel composition to less than about 0 °C.
52. The biofuel composition of claim 51, wherein the fatty acid derivative has a  $\delta^{13}\text{C}$  of from about -10.9 to about -15.4.
53. The biofuel composition of claim 51, further comprising a lower alcohol.
54. A biofuel composition, comprising:  
     at least about 11% of a fatty acid derivative wherein the fatty acid derivative comprises a carbon chain selected from the group consisting of 8:0, 10:0, 12:0, 14:0, 14:1, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3, 20:0, 20:1, 20:2, 20:3, 22:0, 22:1 or 22:3;  
     and  
     at least about 80% conventional diesel fuel.
55. A biofuel composition or feedstock comprising a fatty acid derivative of the formula



wherein X represents  $\text{CH}_3$ ,  $-\text{CH}_2\text{OR}^1$ ;  $-\text{C}(\text{O})\text{OR}^2$ ; or  $-\text{C}(\text{O})\text{NR}^3\text{R}^4$ ;

R is, for each n, independently absent, H or lower alkyl, with at least one R being lower alkyl;

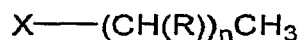
n is an integer from 8 to 34;

$\text{R}^1$ ,  $\text{R}^2$ ,  $\text{R}^3$  and  $\text{R}^4$  independently are selected from H and lower alkyl; and

wherein the fatty acid derivative has a  $\delta^{13}\text{C}$  of from about -10.9 to about -15.4.

56. The biofuel composition of claim 55, wherein the fatty acid derivative is at least about 10% of the biofuel composition.

57. A biofuel composition consisting essentially of a fatty acid derivative of the formula



wherein X represents  $\text{CH}_3$ ,  $-\text{CH}_2\text{OR}^1$ ;  $-\text{C}(\text{O})\text{OR}^2$ ; or  $-\text{C}(\text{O})\text{NR}^3\text{R}^4$ ;

R is, for each n, independently absent, H or lower alkyl;

n is an integer from 8 to 34; and

$\text{R}^1$ ,  $\text{R}^2$ ,  $\text{R}^3$  and  $\text{R}^4$  independently are selected from H and lower alkyl;

wherein the fatty acid derivative has a  $\delta^{13}\text{C}$  of from about -10.9 to about -15.4.

58. A biofuel of any one of claims 38-57, wherein the biofuel comprises less than .1% glycerin.

59. A biofuel of any one of claims 38-58, wherein the biofuel comprises less than 0.1% transesterification catalyst.

FIG. 1

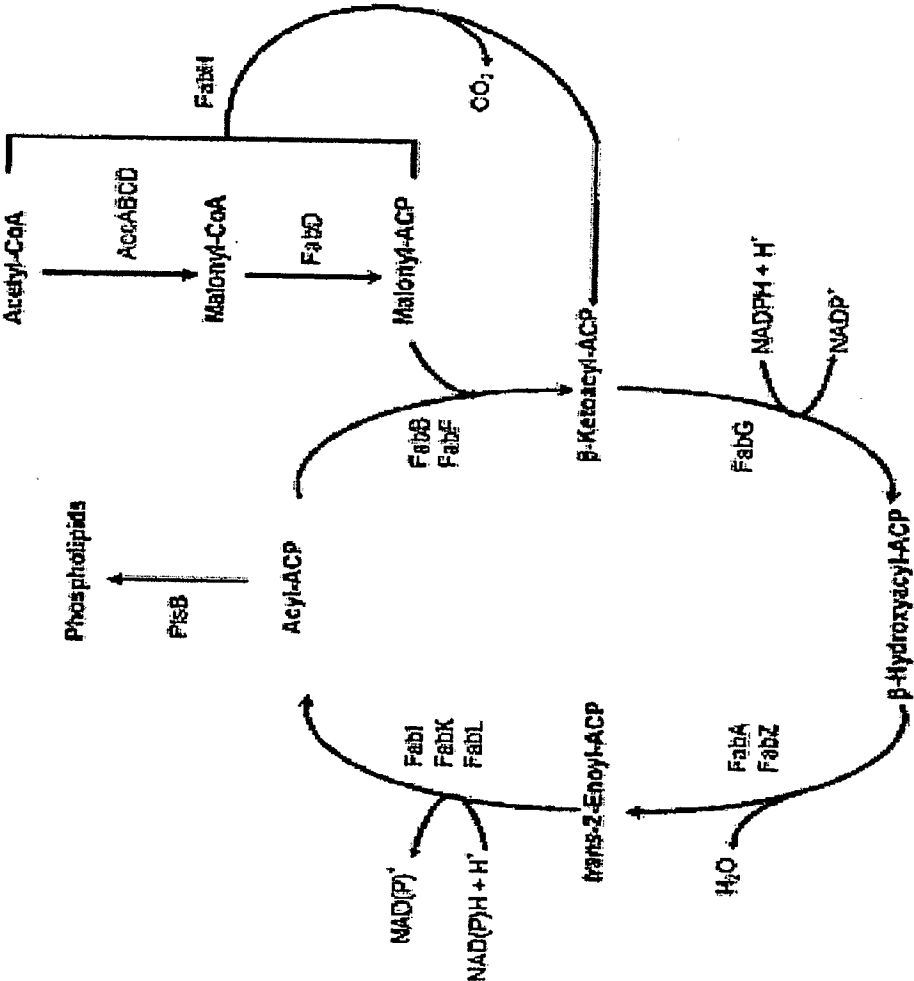
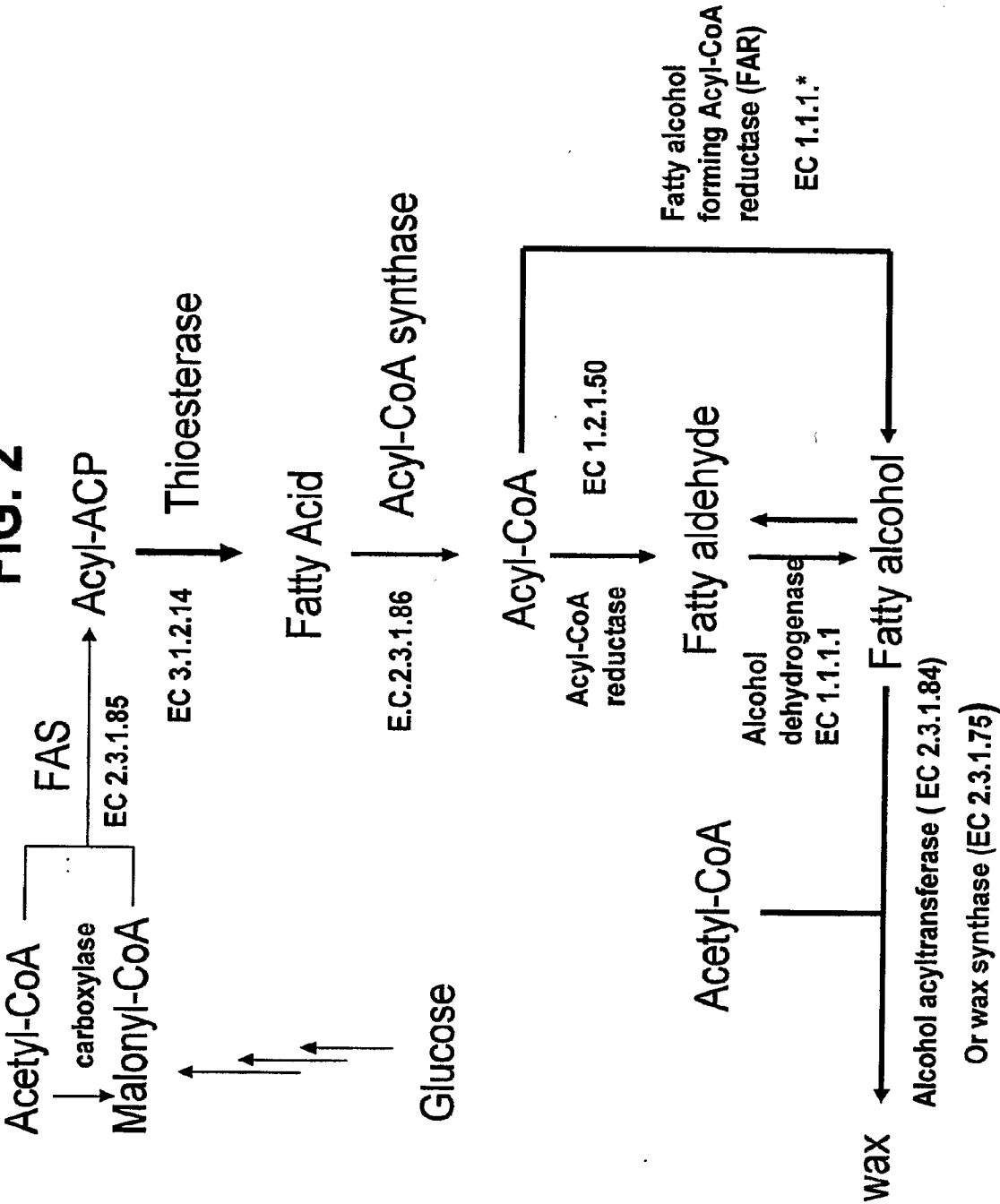


FIG. 2



Fatty alcohol forming acyl-CoA reductase references: Kalscheuer 2006; Metz 2000; Cheng 2004a



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FIG. 3

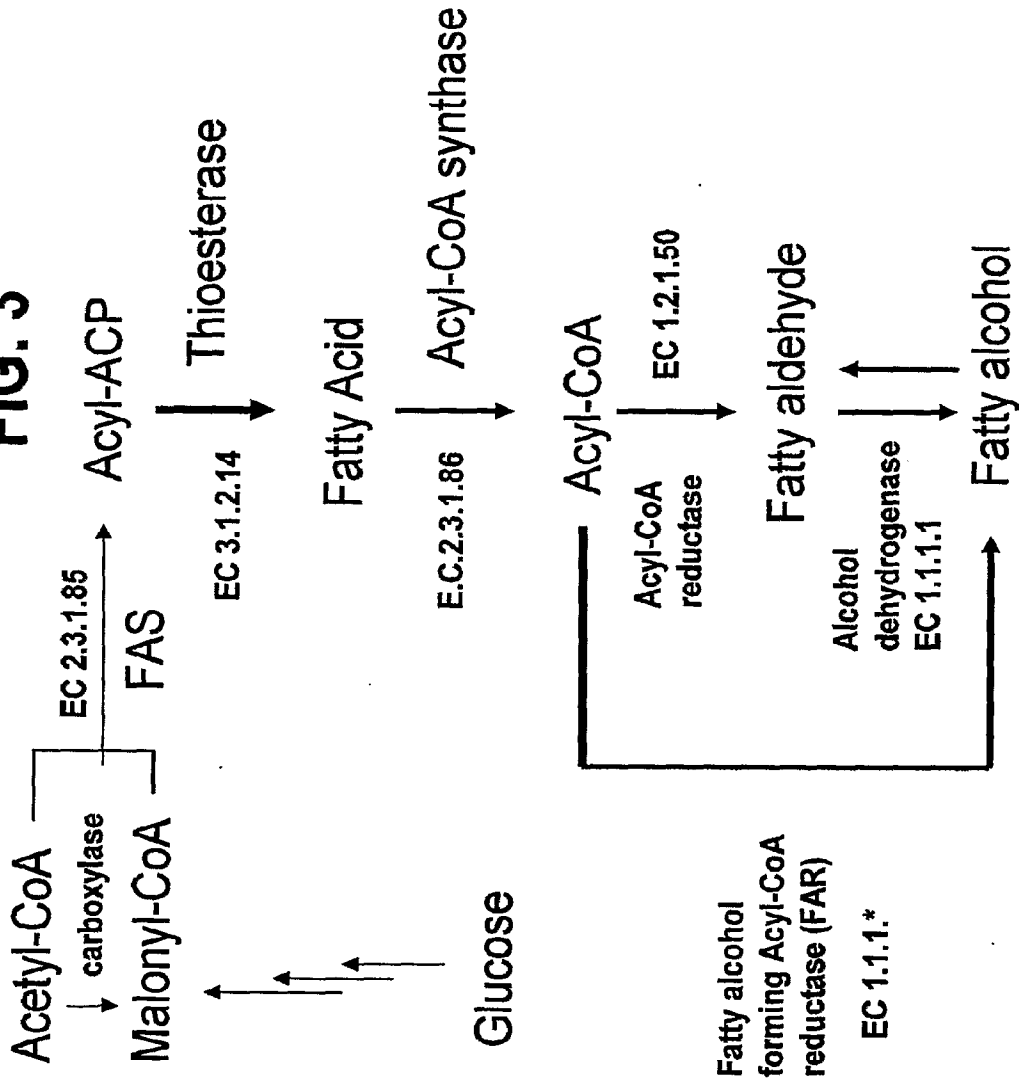
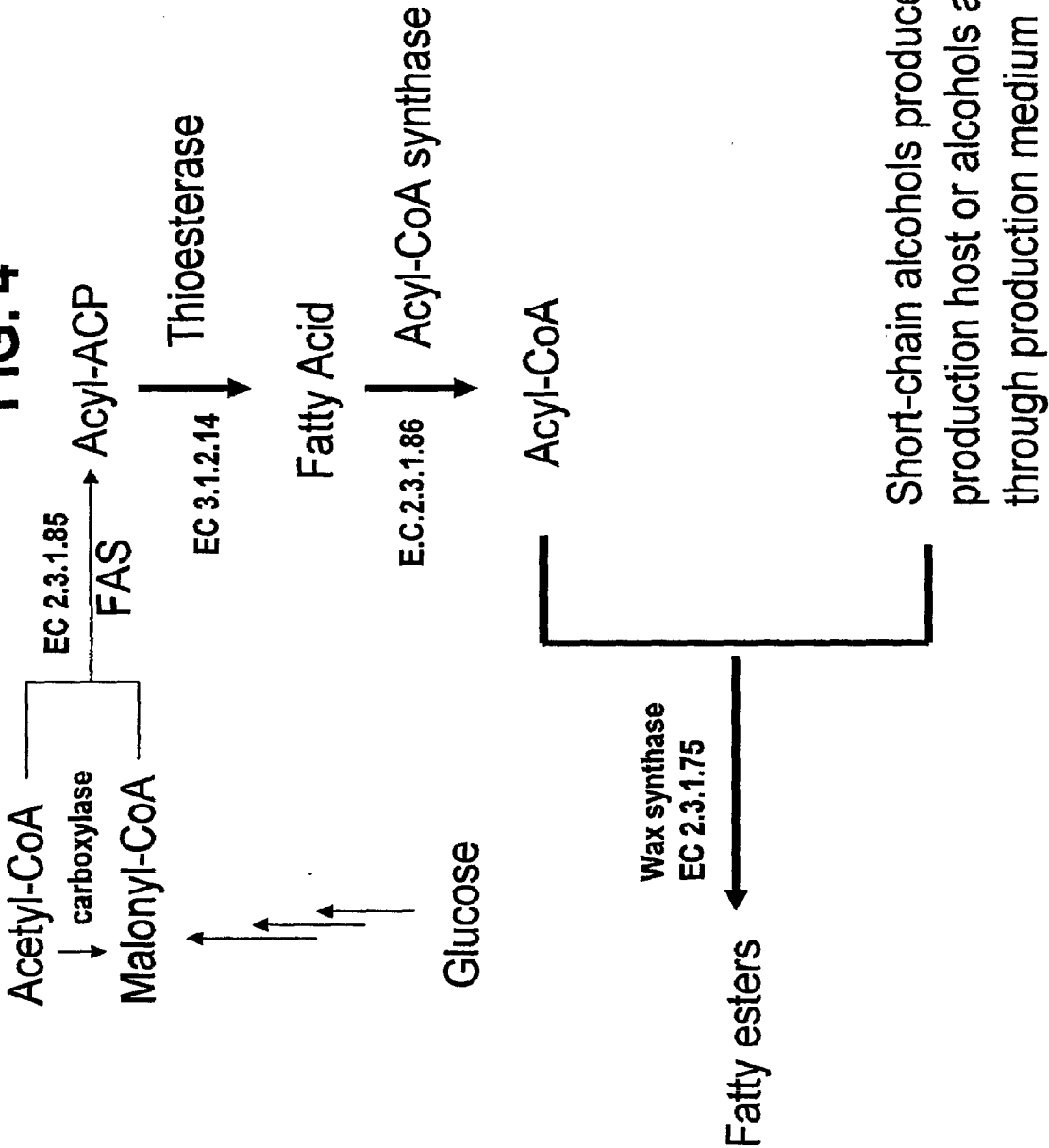
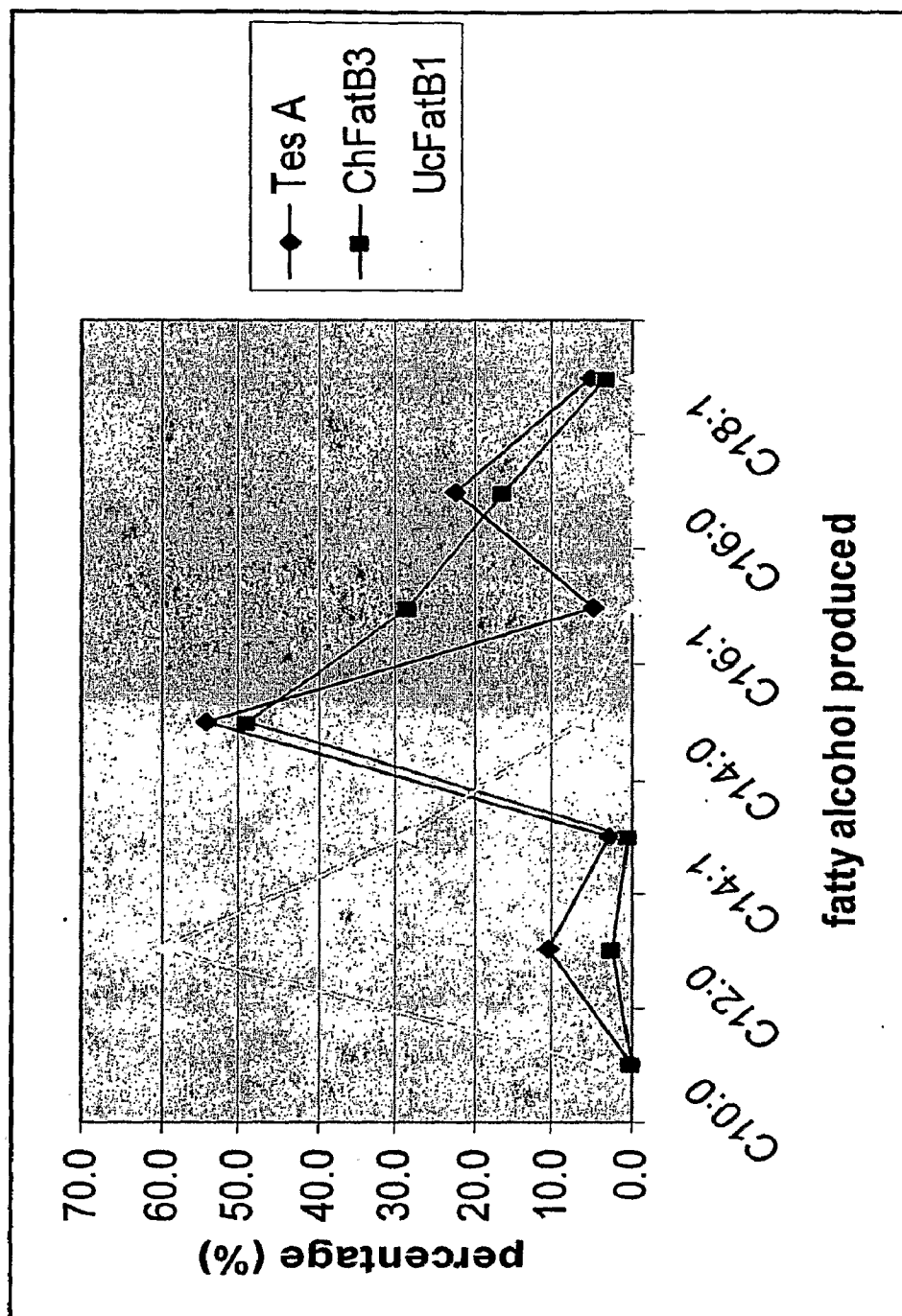


FIG. 4

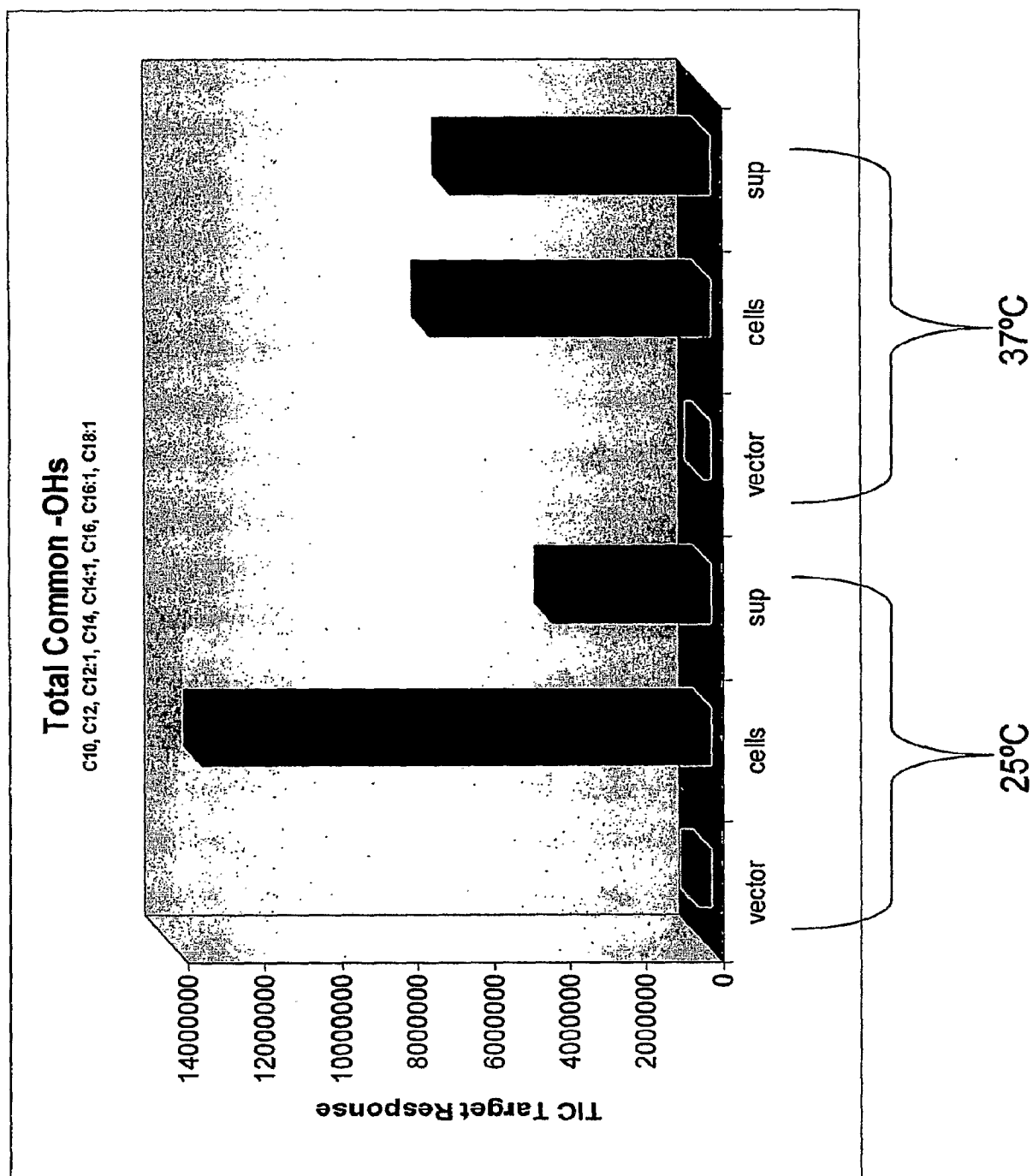


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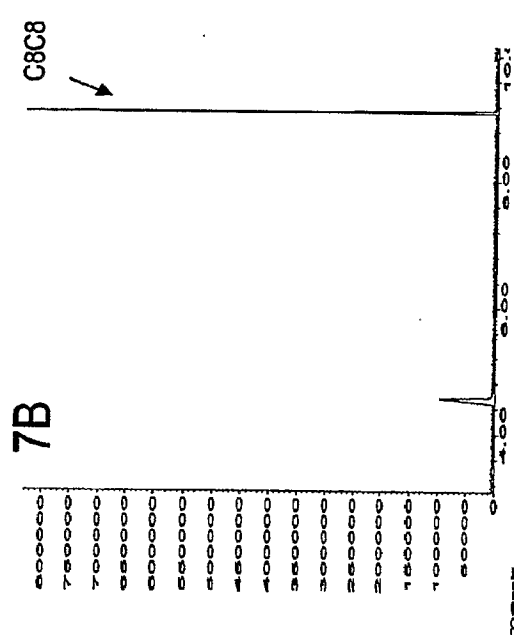
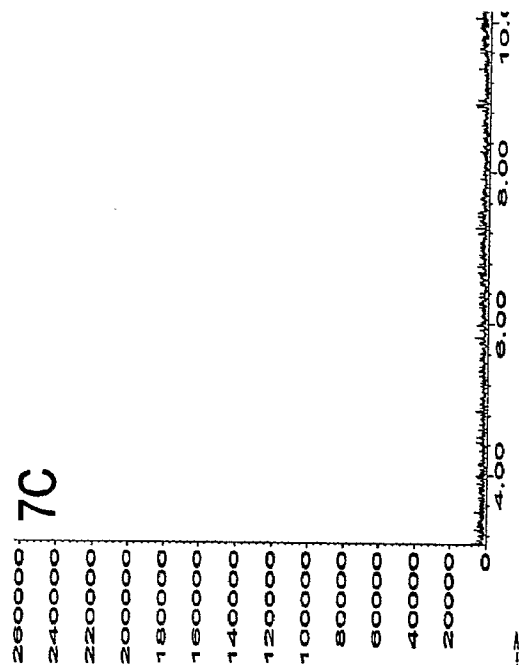
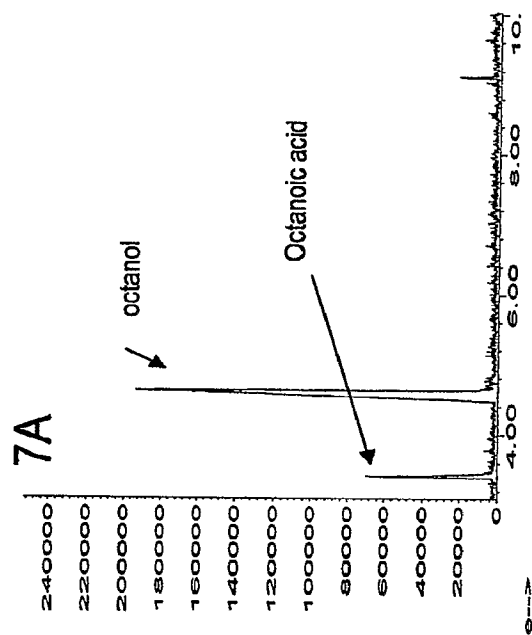
FIG. 5



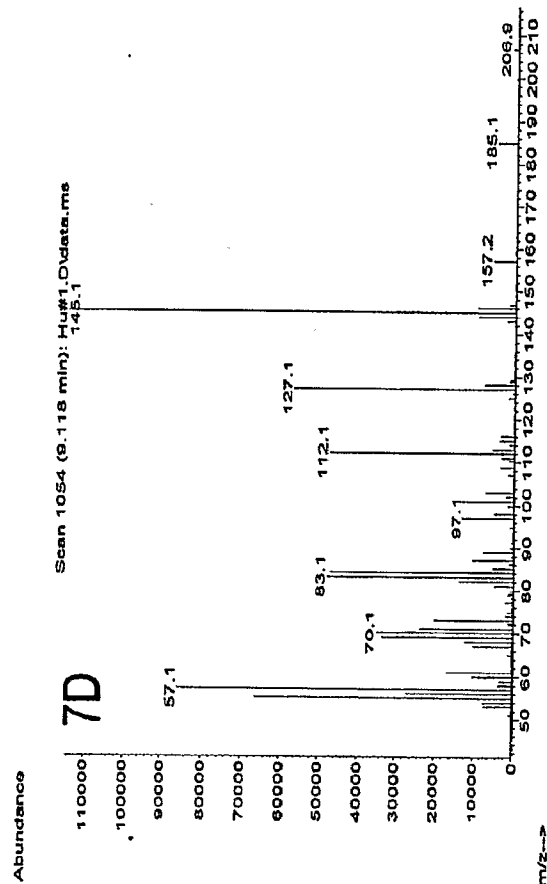
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**FIG. 6**

FIGS. 7A-7D

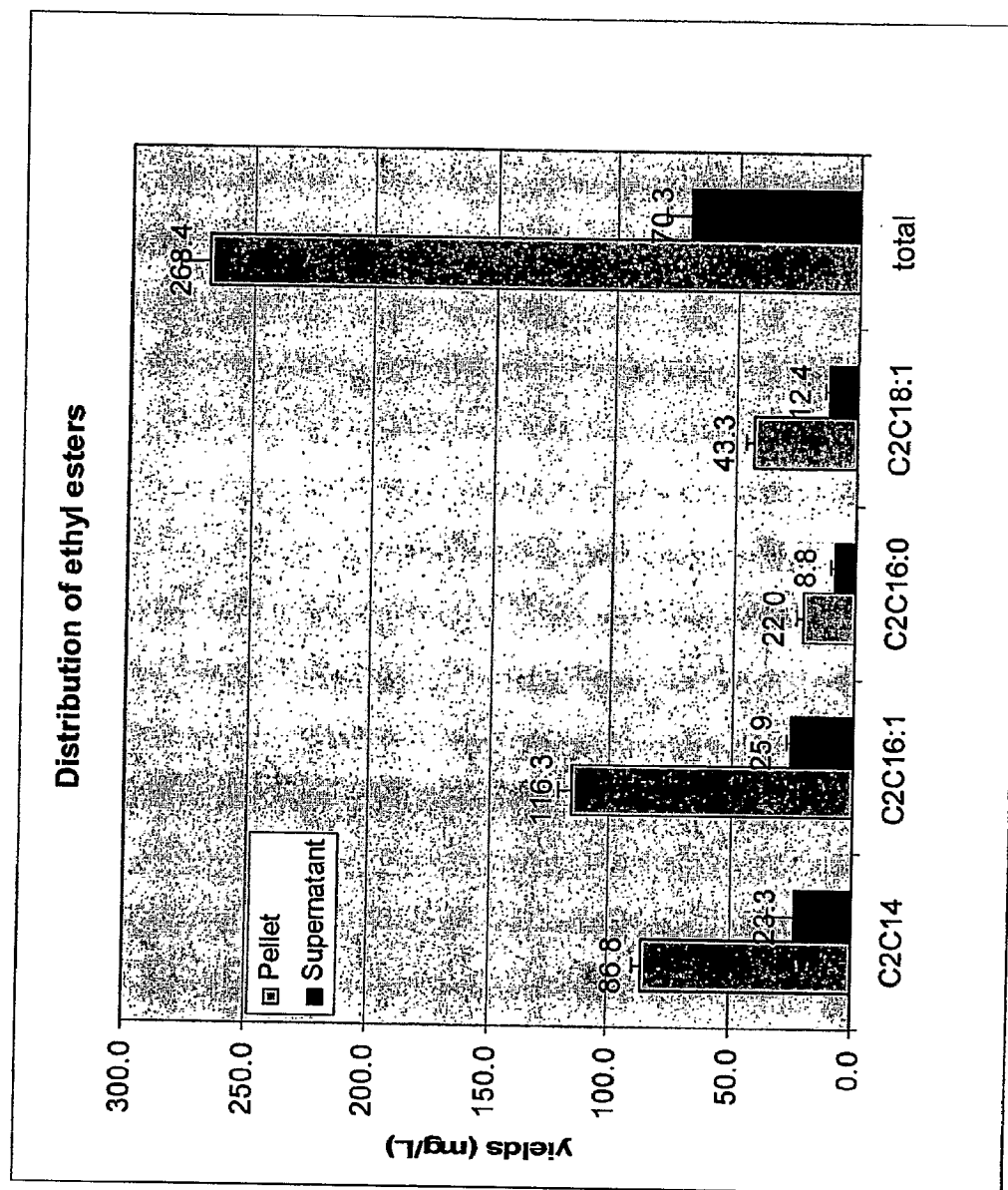


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FIG. 8



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FIG. 9A

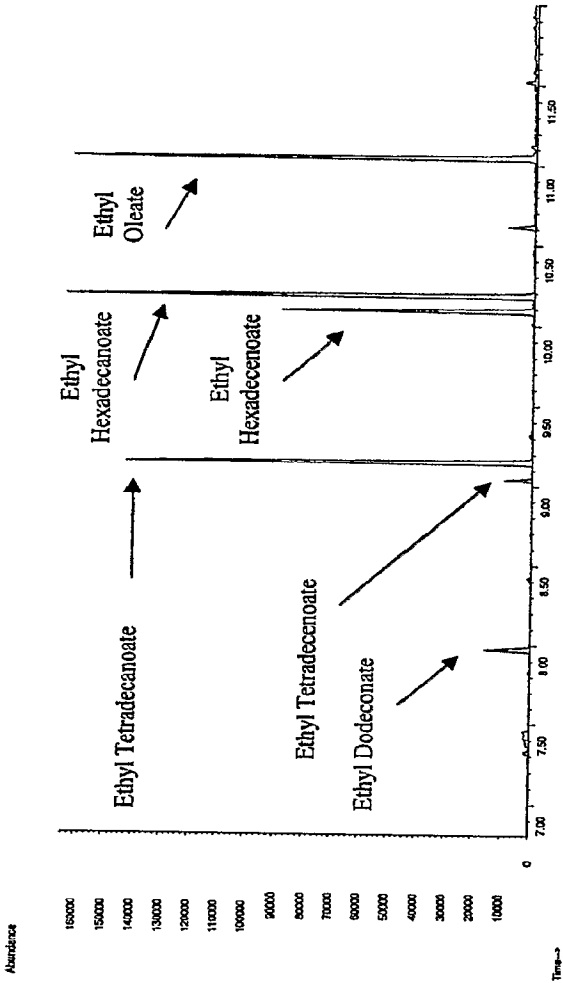
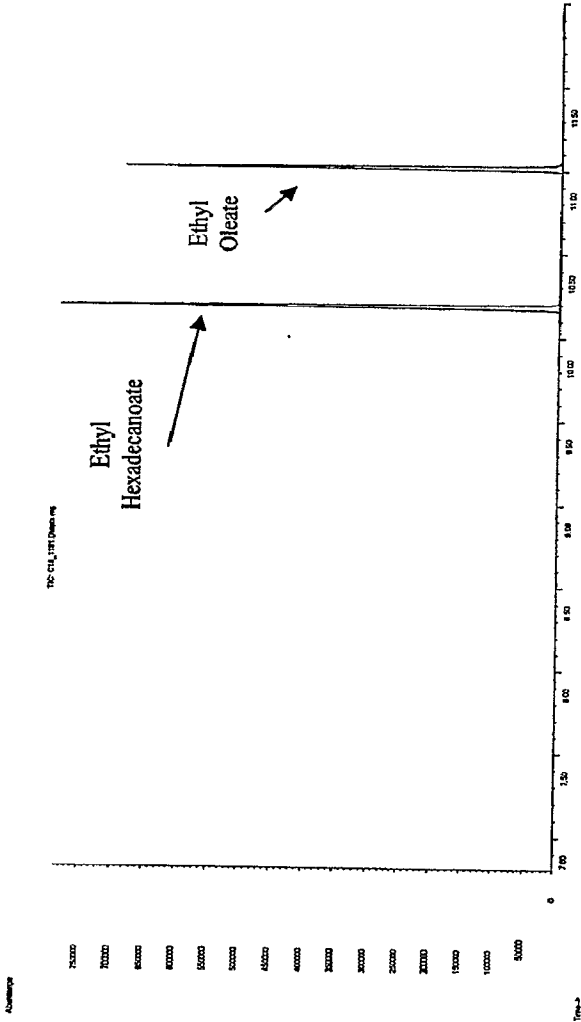


FIG. 9B



**FIG. 10**

Accession Numbers are from NCBI, GenBank, Release 159.0 as of April 15 2007

EC Numbers are from KEGG, Release 42.0 as of April 2007 (plus daily updates up to and including the date for this patent)

<u>CATE</u>	<u>GENE</u>	<u>NAME</u>	<u>EC</u>	<u>ACCESSION NUMBER</u>	<u>MODIFICATION</u>	<u>USE</u>	<u>ORGANISM</u>
<b>1. Fatty Acid Production Increase / Product Production Increase</b>							
		<i>increase acyl-CoA</i>					
		<i>reduce catabolism of derivatives and intermediates</i>					
		<i>reduce feedback inhibition</i>					
		<i>attenuate other pathways that consume fatty acids</i>					
							<b>10/21</b>
accA		Acetyl-CoA carboxylase, subunit		AAC73296, NP_414727	6.4.1.2	Over-express	increase Malonyl-CoA production <i>Escherichia coli</i>
accB		Acetyl-CoA carboxylase, subunit		NP_417721	6.4.1.2	Over-express	increase Malonyl-CoA production <i>Escherichia coli</i>
accC		Acetyl-CoA carboxylase, subunit		NP_417722	6.4.1.2	Over-express	increase Malonyl-CoA production <i>Escherichia coli</i>
accD		Acetyl-CoA carboxylase, subunit		NP_416819	6.4.1.2	Over-express	increase Malonyl-CoA production <i>Escherichia coli</i>
					1.2.4.1,		
aceE		pyruvate dehydrogenase, subunit E1		NP_414656, AAC73226	2.3.1.61,2.3.1. 12	Over-express	increase Acetyl-CoA production <i>Escherichia coli</i>
					1.2.4.1,		
aceF		pyruvate dehydrogenase, subunit E2		NP_414657, AAC73227	2.3.1.61,2.3.1. 12	Over-express	increase Acetyl-CoA production <i>Escherichia coli</i>
ackA		acetate kinase		AAC75356, NP_416799	2.7.2.1	Delete or reduce	increase Acetyl-CoA production <i>Escherichia coli</i> .
ackB		acetate kinase AckB		BAB81430	2.7.2.1	Delete or reduce	increase Acetyl-CoA production <i>Escherichia coli</i>
acpP		acyl carrier protein		AAC74178	NONE	Over-express	increase Acetyl-CoA production <i>Escherichia coli</i>
fadD		acyl-CoA synthase		AP_002424	2.3.1.86	Over-express	increase Fatty acid production <i>Escherichia coli</i> W3110



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adhE	alcohol dehydrogenase	AAC74323, CAA47743	1.1.1.1, 1.2.1.10	Delete or reduce	increase Acetyl-CoA production	<i>Escherichia coli</i> W3111
cer1	Aldehyde decarboxylase	BAA11024	4.1.99.5	Over-express	increase Acetyl-CoA production	<i>Arabidopsis thaliana</i>
fabA	beta-hydroxydecanoyl thioester dehydratase	NP_415474	4.2.1.60	express	fatty acyl-CoA production	<i>E. coli</i> K12
fabD	[acyl-carrier-protein] S-malonyltransferase	AAC74176	2.3.1.39	Over-express	increase Acetyl-CoA production	<i>E. coli</i> K12
fabF	3-oxoacyl-[acyl-carrier-protein] synthase II	AAC74179	2.3.1.179	Delete or OverExpress	increase Acetyl-CoA production	<i>E. coli</i> K12
fabG	3-oxoacyl-[acyl-carrier-protein] reductase	AAC74177	1.1.1.100	Over-express	increase Acetyl-CoA production	<i>E. coli</i> K12
fabH	3-oxoacyl-[acyl-carrier-protein] synthase III	AAC74175	2.3.1.180	Over-express	increase Acetyl-CoA production	<i>E. coli</i> K12
fabI	enoyl-[acyl-carrier-protein] reductase, NADH-dependent	NP_415804	1.3.1.9	express	fatty acyl-CoA production	<i>E. coli</i> K12
fabR	Transcriptional Repressor (3R)-hydroxymyristoyl acyl carrier protein dehydratase	NP_418398	NONE	Delete or reduce	modulate unsaturated fatty acid production	<i>E. coli</i> K12
fabZ		NP_414722	4.2.1.- 1.3.99.3,			<i>E. coli</i> K12
fadE	acyl-CoA dehydrogenase	AAC73325	1.3.99.-	Delete or reduce	increase Acetyl-CoA production	
acr1	Fatty Acyl-CoA reductase	AAC45217	1.2.1.-	Over-express	for fatty alcohol production	<i>E. coli</i> K12
GST	Glutathione synthase	P04425	6.3.2.3	Delete or reduce	increase Acyl-CoA	<i>E. coli</i> K12
gpsA	biosynthetic sn-glycerol 3-phosphate dehydrogenase	AAC76632, NP_418065	EC: 1.1.1.94	Delete or reduce	increase Acetyl-CoA production	<i>E. coli</i> K12
ldhA	lactate dehydrogenase	AAC74462, NP_415898	EC: 1.1.1.28	Delete or reduce	increase Acetyl-CoA production	<i>E. coli</i> K12
Lipase	Triglyceride Lipase	CAA89087, CAA98876	3.1.1.3 4.1.1.9,	express	increase Fatty acid production	<i>E. coli</i> K12
	Malonyl-CoA decarboxylase	AAA26500	4.1.1.41	Over-express		<i>Saccharopolyspora erythraea</i>
panD	aspartate 1-decarboxylase	BAB96708	4.1.1.11	Over-express	increase Acyl-CoA	<i>Escherichia coli</i> W3110
panK a.k.a. coaA	pantothenate kinase	AAC76952	2.7.1.33	Over-express	increase Acetyl-CoA production	

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pdh	Pyruvate dehydrogenase	BAB34380, AAC73227, AAC73226 AAC73989, P09373 AAC77011 AAC73958, NP_415392 AAC75357, NP_416800 CAA46822	1.2.4.1 EC: 2.3.1.54 2.3.1.15 1.2.2.2 2.3.1.8 1.6.1.1	Over-express Delete or reduce D311E mutation Delete or reduce Delete or reduce Over-express	increase Acetyl-CoA production increase Acetyl-CoA production reduce limits on Acyl-CoA pool increase Acetyl-CoA production increase Acetyl-CoA production conversion NADH to NADPH or vice versa	<i>E. coli</i> K12
pflB	formate acetyltransferase					
pflB	acyltransferase					
poxB	pyruvate oxidase					
pta	phosphotransacetylase					
udhA	pyridine nucleotide transhydrogenase					
	fused 3-hydroxybutyryl-CoA epimerase/delta(3)-cis-delta(2)-trans enoyl-CoA isomerase/enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase					
fadB	3-hydroxyacyl-CoA dehydrogenase; K01692 enoyl-CoA hydratase; K01782 3-hydroxybutyryl-CoA epimerase	AP_003956	1.1.1.35	Delete or reduce	Block fatty acid degradation	<i>E. coli</i>
fadJ						
fadA	3-ketoacyl-CoA thiolase	AAC75401 BAE77458	1.1.1.35, 4.2.1.17, 5.1.2.3 2.3.1.16	Delete or reduce Delete or reduce	Block fatty acid degradation Block fatty acid degradation	<i>E. coli</i> <i>E. coli</i>
fadI	beta-ketoacyl-CoA thiolase	AAC75402	1.5.1.29, 1.16,	Delete or reduce	Block fatty acid degradation	<i>E. coli</i>
YdiO	acyl-coA dehydrogenase	YP_852786	1.3.99.-	Delete or reduce	Block fatty acid degradation	<i>E. coli</i>
<u>2. Structure Control</u>						
<u>2A. Chain Length Control</u>						
2	tesA	P0ADA1	3.1.2.-	Delete 1 and express	C18 Chain Length	
	tesA without leader sequence	AAC73596, NP_415027	3.1.1.-	express or overexpress	C18:1	<i>E. coli</i> <i>Umbellularia</i> <i>californica</i>
	fatB	Q41635	3.1.1.-	express or overexpress	C12:0	
	(umbellularia) fatB2	AAC49269	3.1.1.-	express or overexpress	C8:0 - C10:0	<i>Cuphea hookeriana</i>
	(umbellularia)					

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fatB3	thioesterase	AAC72881	3.1.1.1.-	express or overexpress	C14:0 - C16:0	<i>Cuphea hookeriana</i>
fatB (cinnamonomum)	thioesterase	Q39473	3.1.1.1.-	express or overexpress	C14:0	<i>Cinnamomum camphora</i>
fatB[M141T]* fatA1 (Helianthus)	thioesterase	CAA85388	3.1.1.1.-	express or overexpress	C16:1	<i>Arabidopsis thaliana</i>
atfata fatA	thioesterase	AAL79361 NP 189147, NP 193041 CAC39106	3.1.1.1.-	express or overexpress	C18:1	<i>Helianthus annuus</i>
fatA (cuphea)	thioesterase	AAC72883	3.1.1.1.-	express or overexpress	C18:1	<i>Arabidopsis thaliana</i> <i>Brassica juncea</i> <i>Cuphea hookeriana</i>

2B. Branching Control

attenuate *FabH*  
express *FabH*  
from *S.*  
*glaucescens* and  
knock out  
endogenous  
*FabH*  
express *FabH*  
from *B. subtilis*  
and knock out  
endogenous  
*FabH*  
bdk - E3 -  
dihydrodiplopyl  
dehydrogenase  
subunit  
bkd - E1 -  
alpha/beta  
subunit

increase branched chain fatty acid  
derivatives

EC 1.2.4.4

EC 1.2.4.4



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bkdC	dihydrolipoyl transacetylase (E2)	AAA65617	EC 1.2.4.4	express or Over-Express	make branched-chain acyl-CoA precursors	<i>Pseudomonas putida</i>
lpd	dihydrolipoamide dehydrogenase (E3)	NP_414658	1.8.1.4	express or Over-Express	make branched-chain acyl-CoA precursors	<i>Escherichia coli</i>
IlvE	branched-chain amino acid aminotransferase	YP_026247	2.6.1.42	express or Over-Express	make branched $\alpha$ -ketoacids	<i>Escherichia coli</i>
IlvE	branched-chain amino acid aminotransferase	AAF34406	2.6.1.42	express or Over-Express	make branched $\alpha$ -ketoacids	<i>Lactococcus lactis</i>
IlvE	branched-chain amino acid aminotransferase	NP_745648	2.6.1.43	express or Over-Express	make branched $\alpha$ -ketoacids	<i>Pseudomonas putida</i>
IlvE	branched-chain amino acid aminotransferase	NP_629657	2.6.1.42	express or Over-Express	make branched $\alpha$ -ketoacids	<i>Streptomyces coelicolor</i>
ccr	crotonyl-CoA reductase	NP_630556	1.1.1.9	express or Over-Express	Converting crotonyl-CoA to butyryl-CoA	<i>Streptomyces coelicolor</i>
ccr	crotonyl-CoA reductase	AAD53915	1.1.1.9	express or Over-Express	Converting crotonyl-CoA to butyryl-CoA	<i>Streptomyces cinnamomensis</i>
IcmA, isobutyryl-CoA mutase	isobutyryl-CoA mutase, subunit A	NP_629554	5.4.99.2	express or Over-Express	converting butyryl-CoA to isobutyryl-CoA	<i>Streptomyces coelicolor</i>
IcmA, isobutyryl-CoA mutase	isobutyryl-CoA mutase, subunit A	AAC08713	5.4.99.2	express or Over-Express	converting butyryl-CoA to isobutyryl-CoA	<i>Streptomyces cinnamomensis</i>
IcmB, isobutyryl-CoA mutase	isobutyryl-CoA mutase, subunit B	NP_630904	5.4.99.13	express or Over-Express	converting butyryl-CoA to isobutyryl-CoA	<i>Streptomyces coelicolor</i>
IcmB, isobutyryl-CoA mutase	isobutyryl-CoA mutase, subunit B	AIJ246005	5.4.99.13	express or Over-Express	converting butyryl-CoA to isobutyryl-CoA	<i>Streptomyces cinnamomensis</i>
FabH, ACPs and fabF genes with specificity for branched chain acyl-CoAs						
IlvE		CAC12788	EC2.6.1.42	over express	branched chain amino acid amino transferase	<i>S. carnosus</i>
FabH1	beta-ketoacyl-ACP synthase III	NP_626634	2.3.1.180	express or Over-Express	initiation of branched-chain fatty acid biosynthesis	<i>Streptomyces coelicolor</i>

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ACP	acyl-carrier protein	NP_626635	NONE	express or Over-Express	initiation and elongation of branched-chain fatty acid biosynthesis	<i>Streptomyces coelicolor</i>
FabF	beta-ketoacyl-ACP synthase II	NP_626636	2.3.1.179	express or Over-Express	elongation of branched-chain fatty acid biosynthesis	<i>Streptomyces coelicolor</i>
FabH3	beta-ketoacyl-ACP synthase III	NP_823466	2.3.1.180	express or Over-Express	initiation of branched-chain fatty acid biosynthesis	<i>Streptomyces avermitilis</i>
FabC3 (ACP)	acyl-carrier protein	NP_823467	NONE	express or Over-Express	initiation and elongation of branched-chain fatty acid biosynthesis	<i>Streptomyces avermitilis</i>
FabF	beta-ketoacyl-ACP synthase II	NP_823468	2.3.1.179	express or Over-Express	elongation of branched-chain fatty acid biosynthesis	<i>Streptomyces avermitilis</i>
FabH_A	beta-ketoacyl-ACP synthase III	NP_389015	2.3.1.180	express or Over-Express	initiation of branched-chain fatty acid biosynthesis	<i>Bacillus subtilis</i>
FabH_B	beta-ketoacyl-ACP synthase III	NP_388898	2.3.1.180	express or Over-Express	initiation of branched-chain fatty acid biosynthesis	<i>Bacillus subtilis</i>
ACP	acyl-carrier protein	NP_389474	NONE	express or Over-Express	initiation and elongation of branched-chain fatty acid biosynthesis	<i>Bacillus subtilis</i>
FabF	beta-ketoacyl-ACP synthase II	NP_389016	2.3.1.179	express or Over-Express	elongation of branched-chain fatty acid biosynthesis	<i>Bacillus subtilis</i>
SmaIDRAFT_08 18	beta-ketoacyl-ACP synthase III	ZP_01643059	2.3.1.180	express or Over-Express	initiation of branched-chain fatty acid biosynthesis	<i>Stenotrophomonas maltophilia</i>
SmaIDRAFT_08 21	acyl-carrier protein	ZP_01643063	NONE	express or Over-Express	initiation and elongation of branched-chain fatty acid biosynthesis	<i>Stenotrophomonas maltophilia</i>
SmaIDRAFT_08 22	beta-ketoacyl-ACP synthase II	ZP_01643064	2.3.1.179	express or Over-Express	elongation of branched-chain fatty acid biosynthesis	<i>Stenotrophomonas maltophilia</i>
FabH	beta-ketoacyl-ACP synthase III	YP_123672	2.3.1.180	express or Over-Express	initiation of branched-chain fatty acid biosynthesis	<i>Legionella pneumophila</i>
ACP	acyl-carrier protein	YP_123675	NONE	express or Over-Express	initiation and elongation of branched-chain fatty acid biosynthesis	<i>Legionella pneumophila</i>
FabF	beta-ketoacyl-ACP synthase II	YP_123676	2.3.1.179	express or Over-Express	elongation of branched-chain fatty acid biosynthesis	<i>Legionella pneumophila</i>
FabH	beta-ketoacyl-ACP synthase III	NP_415609	2.3.1.180	delete or reduce	initiation of branched-chain fatty acid biosynthesis	<i>Escherichia coli</i>

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FabF	beta-ketoacyl-ACP synthase II	NP_415613	2.3.1.179	delete or reduce	elongation of branched-chain fatty acid biosynthesis	<i>Escherichia coli</i>
<i>To Produce Cyclic Fatty Acids</i>						
AnsJ	dehydratase (putative)	not available	not available	express or Over-Express	cyclohexylcarbonyl-CoA biosynthesis	<i>Streptomyces collinus</i>
AnsK	CoA ligase (putative)	not available	not available	express or Over-Express	cyclohexylcarbonyl-CoA biosynthesis	<i>Streptomyces collinus</i>
AnsL	dehydrogenase (putative)	not available	not available	express or Over-Express	cyclohexylcarbonyl-CoA biosynthesis	<i>Streptomyces collinus</i>
ChcA	enoyl-CoA reductase	U72144	E1.3.1.34	express or Over-Express	cyclohexylcarbonyl-CoA biosynthesis	<i>Streptomyces collinus</i>
AnsM	oxidorecutase (putative)	not available	not available	express or Over-Express	cyclohexylcarbonyl-CoA biosynthesis	<i>Streptomyces collinus</i>
PlmJ	dehydratase (putative)	AAQ84158	not available	express or Over-Express	cyclohexylcarbonyl-CoA biosynthesis	<i>Streptomyces sp. HK803</i>
PlmK	CoA ligase (putative)	AAQ84158	not available	express or Over-Express	cyclohexylcarbonyl-CoA biosynthesis	<i>Streptomyces sp. HK803</i>
PlmL	dehydrogenase (putative)	AAQ84159	not available	express or Over-Express	cyclohexylcarbonyl-CoA biosynthesis	<i>Streptomyces sp. HK803</i>
ChcA	enoyl-CoA reductase	AAQ84160	E1.3.1.34	express or Over-Express	cyclohexylcarbonyl-CoA biosynthesis	<i>Streptomyces sp. HK803</i>
PlmM	oxidorecutase (putative)	AAQ84161	not available	express or Over-Express	cyclohexylcarbonyl-CoA biosynthesis	<i>Streptomyces sp. HK803</i>
ChcB	enoyl-CoA isomerase	AF268489	not available	express or Over-Express	cyclohexylcarbonyl-CoA biosynthesis	<i>Streptomyces collinus</i>
ChcB/CaiD	enoyl-CoA isomerase	NP_629292	4.2.1.-	express or Over-Express	cyclohexylcarbonyl-CoA biosynthesis	<i>Streptomyces coelicolor</i>
ChcB/CaiD	enoyl-CoA isomerase	NP_824296	4.2.1.-	express or Over-Express	cyclohexylcarbonyl-CoA biosynthesis	<i>Streptomyces avermitilis</i>
<u>2C. Saturation Level Control</u>						
Sfa	Suppressor of FabA	AAN79592, AAC44390	Can't find	Over-express	increase monounsaturated fatty acids	<i>E.coli</i>
also see FabA in sec. I				express	produce unsaturated fatty acids	
GnsA	suppressors of the secG null mutation	ABD18647.1	NONE	Over-express	increase unsaturated fatty acid esters	<i>E.coli</i>

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GnsB	suppressors of the secG null mutation	AAC74076.1	NONE	Over-express	increase unsaturated fatty acid esters	<i>E.coli</i>
also see section 2A - items with :0 are unsaturated (no double bonds) and with :1 are saturated (1 double bond)						
fabB	3-oxoacyl-[acyl-carrier-protein] synthase I	BAA16180	EC:2.3.1.41	overexpress	modulate unsaturated fatty acid production	<i>Escherichia coli</i>
fabK	trans-2-enoyl-ACP reductase II	AAF98273	1.3.1.9	express	modulate unsaturated fatty acid production	<i>Streptococcus pneumoniae</i> <i>Bacillus</i>
fabL	enoyl-(acyl carrier protein) reductase	AAU39821	1.3.1.9	express	modulate unsaturated fatty acid production	<i>licheniformis DSM 13</i>
fabM	trans-2, cis-3-decenoyl-ACP isomerase	DAA05501	5.3.3.14	Over-express	modulate unsaturated fatty acid production	<i>Streptococcus mutans</i>
<u>3. Final Product Output</u>						
<u>3A. Wax Output</u>						
AT3G51970	long-chain-alcohol O-fatty-acyltransferase	NP_190765	2.3.1.75	express	wax production	<i>Arabidopsis thaliana</i>
	thioesterase (see chain length control section)		3.1.2.14	express	increase fatty acid production	
	fatty alcohol forming acyl-CoA reductase		1.1.1.*	express	convert acyl-coa to fatty alcohol	
acr1	acyl-CoA reductase (ACR1)	YP_047869	1.2.1.50	express	convert acyl-coa to fatty alcohol	<i>Acinetobacter sp. ADP1</i>
yqhD	alcohol dehydrogenase	AP_003562	1.1.1.1	express	increase	<i>E. coli W3110</i>
ELO1	Fatty acid elongase	BAD98251	2.3.1.74	express	produce very long chain length fatty acids	<i>Pichia angusta</i>
plsC	acyltransferase	AAA16514	2.3.1.-	express		<i>Saccharomyces cerevisiae</i>
DAGAT	diacylglycerol acyltransferase	AAF19262	2.3.1.20	express	wax production	<i>Arabidopsis thaliana</i>



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hWS	acyl-CoA wax alcohol acyltransferase bifunctional wax ester synthase/acyl-CoA:diacylglycerol acyltransferase	AAx48018	Can't find	express	wax production	<i>Homo sapiens</i>
af1		AAO17391	2.3.1.20	express	wax production	<i>Acinetobacter</i> sp. ADP1
mWS	wax ester synthase (simmondsia)	AAD38041	2.3.1.75	express	wax production	<i>Simmondsia</i> <i>chinensis</i>
<u>3B. Fatty Alcohol Output</u>						
	various thioesterases (refer to Sec. 2A)		3.1.2.14	express	produce	
acr1	acyl-CoA reductase	YP_047869	1.2.1.50	express	produce	<i>Acinetobacter</i> sp. ADP1
yqhD	alcohol dehydrogenase	AP_003562	1.1.1.1	express	produce	<i>Escherichia coli</i> W3110
BmFAR	FAR (fatty alcohol forming acyl-CoA reductase)	BAC79425	1.1.1.*	express	reduce fatty acyl-CoA to fatty alcohol	<i>Bombyx mori</i>
Akr1a4	Mammalian microsomal aldehyde reductase	NP_067448	1.1.1.21	express	produce	<i>Mus musculus</i> <i>Geobacillus</i> <i>thermodenitrificans</i> NG80-2 <i>E. Coli</i> K12
GTNG_1865	Long-chain aldehyde dehydrogenase	YP_00112597		express	produce	
FadD	acyl-CoA synthetase	NP_416319	EC 6.2.1.3	express	produce more	
<i>To make Butanol</i>						
atoB	acetyl-CoA acetyltransferase	YP_049388	2.3.1.9	express	produce	<i>Erwinia carotovora</i>
hbd	Beta-hydroxybutyryl-CoA dehydrogenase	BAD51424	1.1.1.157	express	produce	<i>Butyrivibrio</i> <i>fibrisolvens</i> <i>Clostridium</i> <i>perfringens</i> <i>Clostridium</i> <i>beijerinckii</i> <i>Clostridium</i> <i>beijerinckii</i> <i>Escherichia coli</i> CFT073
CPE0095	crotonase	BAB79801	4.2.1.17	express	produce	
bed	butyryl-CoA dehydrogenase	AAM14583	Can't find	express	produce	
ALDH	CoA-acylating dehydrogenase	AAT66436	Can't find	express	produce	
AdhE	aldehyde-alcohol dehydrogenase	AAN80172	1.1.1.1 1.2.1.10	express	produce	

3C. Fatty Acid Ester Output

thioesterase	see chain length control section	3.1.2.14	express	produce	<i>Acinetobacter sp.</i>
acrI	acyl-CoA reductase	YP_047869	express	produce	<i>ADP-I</i>
yqhD	alcohol dehydrogenase	AP_003562	express	produce	<i>E. Coli K12</i>
AAT	alcohol O-acetyltransferase	AAG13130	express	produce	<i>Fragaria x ananassa</i>

4. Export

Wax ester  
exporter (FATP  
family, Fatty  
Acid (long chain)  
Transport  
Protein)

ABC transporter	putative alkane transporter	NP_524723	NONE	express	export wax	<i>Drosophila melanogaster</i>
		AAN73268 Atlg51500, AY734542, Atlg21090, Atlg51460	NONE	express	export products	<i>Rhodococcus erythropolis</i>
CER5	wax transporter	NP_171908	NONE	express	export products	<i>Arabidopsis thaliana</i>
AtMRP5	Arabidopsis thaliana multidrug resistance-associated	JCS491	Can't Find	express	export products	<i>Arabidopsis thaliana</i>
AmiS2	ABC transporter AmiS2	NP_181228	NONE	express	export products	<i>Rhodococcus sp.</i>
APGP1	ARABIDOPSIS THALIANA P GLYCOPROTEIN1	CAF23274	NONE	express	export products	<i>Arabidopsis thaliana</i>
AcrA	putative multidrug-efflux transport protein acrA	CAF23275	NONE	express	export products	<i>Candidatus</i>
AcrB	probable multidrug-efflux transport protein, acrB	ABD59001	NONE	express	export products	<i>Protochlamydia amoebophila UWE25</i>
ToIC	Outer membrane protein [Cell envelope biogenesis,			express	export products	<i>Candidatus</i>
						<i>Protochlamydia amoebophila UWE25</i>
						<i>Francisella tularensis subsp. novicida</i>

AcrE AcrF	transmembrane protein affects septum formation and cell membrane permeability Acriflavine resistance protein F	YP_312213	NONE	express	export products	<i>Shigella sonnei</i> Ss046
		P24181	NONE	express	export products	<i>Escherichia coli</i>
		NP_682408.1		express	export products	<i>Thermosynechococcus elongatus BP-1J</i>
tl1618	multidrug efflux transporter	NP_682409.1		express	export products	<i>Thermosynechococcus elongatus BP-1J</i>
tl1619	multidrug efflux transporter	NP_680930.1		express	export products	<i>Thermosynechococcus elongatus BP-1J</i>
tl10139	multidrug efflux transporter					
<u>5. Fermentation</u>						
umuD umuC NADH:NADPH transhydrogenase (alpha and beta subunits)	replication checkpoint genes  DNA polymerase V, subunit DNA polymerase V, subunit NADH:NADPH transhydrogenase (alpha and beta subunits)	YP_310132	3.4.21.-	Over-express	increase output efficiency	<i>Shigella sonnei</i> Ss046
		ABC42261	3.4.21.-	Over-express	increase output efficiency	<i>Escherichia coli</i>
		<u>P07001</u> , <u>P0AB70</u>	1.6.1.1. 1.6.1.2	express	increase output efficiency	<i>Shigella flexneri</i>

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\*\* END OF SHEET \*\*

# A cobalt-porphyrin enzyme converts a fatty aldehyde to a hydrocarbon and CO

(decarbonylase/*Botryococcus braunii*/solubilization)

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**ABSTRACT** The final step in hydrocarbon biosynthesis involves loss of CO from a fatty aldehyde. This decarbonylation is catalyzed by microsomes from *Botryococcus braunii*. Among the several detergents tested for solubilizing the decarbonylase, octyl  $\beta$ -glucoside (0.1%) was found to be the most effective and released 65% of the enzyme activity in soluble form. FPLC of the solubilized enzyme preparation with Superose 6 followed by ion-exchange FPLC with Mono Q resulted in 200-fold increase in specific activity with 7% recovery. The purified enzyme released nearly 1 mol of CO for each mol of hydrocarbon. SDS/PAGE of the enzyme preparation showed two protein bands of equal intensity at 66 and 55 kDa. The absorption spectrum of the enzyme with bands at 410 nm, 425 nm, 580 nm, and 620 nm suggests the presence of a porphyrin. Electron microprobe analysis revealed that the enzyme contained Co. Purification of the decarbonylase from *B. braunii* grown in  $^{57}\text{CoCl}_2$  showed that  $^{57}\text{Co}$  coeluted with the decarbonylase. These results suggest that the enzyme contains Co that might be part of a Co-porphyrin, although a corrin structure cannot be ruled out. Co-protoporphyrin IX itself caused decarbonylation of octadecanal at 60°C, whereas the metal ion or protoporphyrin alone, or several other metal porphyrins, did not cause decarbonylation. These results strongly suggest that biosynthesis of hydrocarbons is effected by a microsomal Co-porphyrin-containing enzyme that catalyzes decarbonylation of aldehydes and, thus, reveal a biological function for Co in plants.

Aliphatic nonisoprenoid hydrocarbons are ubiquitous in living organisms in both the plant and animal kingdoms (1, 2). Widespread occurrence of hydrocarbons in animals, accumulation of hydrocarbons under pathological conditions (3, 4), demonstrated biosynthesis of hydrocarbons in mammalian nerve tissue (2), and decreased hydrocarbon synthesis associated with neurological disorders (5, 6) suggest important biological functions for this class of simple compounds.

On the basis of the results obtained with specifically labeled precursors in higher plant tissues, it was proposed that *n*-hydrocarbons are produced by elongation of a fatty acid followed by the loss of the carboxyl carbon (7–9). Subsequent studies with insects (10) and mammals (2) supported this mechanism for alkane biosynthesis. Microsomal preparations from plant and animal tissues that generate alkanes have been shown to catalyze elongation of fatty acids (11, 12). The nature of the reaction that results in the loss of the carboxyl carbon remained obscure as the chemical nature of the immediate precursors of hydrocarbon was unknown until recently. Aldehydes with one carbon more than the alkanes were found to accumulate when hydrocarbon synthesis was inhibited by thiol compounds such as dithioerythritol (DTE) (13). In cell-free preparations that generate hydrocarbons, the observation that an aldehyde with one car-

bon more than the hydrocarbon was formed suggests that the aldehyde might be the immediate precursor of hydrocarbons (14). However, the aldehyde was not recognized as an intermediate in alkane synthesis because a plausible mechanism for conversion of an aldehyde to an alkane was not obvious until the discovery that porphyrin-coordinated Ru complexes catalyze the conversion of aldehydes to corresponding alkanes with the loss of CO (15, 16). This laboratory has demonstrated that particulate preparations from young pea leaves (17), the uropygial gland of the eared grebe (18), and diapausing flesh flies (19) catalyzed decarbonylation of aldehydes to yield the corresponding alkanes. However, the enzyme that catalyzes this biochemical reaction had not been solubilized and purified and, thus, the nature of the enzyme and the mechanism of the reaction remained obscure.

The green colonial alga *Botryococcus braunii*, race A, produces linear odd-numbered  $\text{C}_{27}$ ,  $\text{C}_{29}$ , and  $\text{C}_{31}$  hydrocarbons that total up to 32% of the alga's dry weight (20–22). Microsomal preparations from this organism catalyze the decarbonylation of an aldehyde to alkane (23). In this paper, we report the solubilization and purification of the aldehyde decarbonylase and present data that suggest that this enzyme contains a Co-porphyrin and, thus, reveal a possible biological function for Co in plants.

## MATERIALS AND METHODS

**Chemicals.** [1- $^{14}\text{C}$ ]Octadecanoic acid and *cis*-9-[9,10- $^3\text{H}$ ]octadecenoic acid were from DuPont/New England Nuclear.  $^{57}\text{CoCl}_2$  was obtained from Amersham. Pyridinium chlorochromate, chloroplatinic acid, and  $\text{RhCl}_3 \cdot \text{H}_2\text{O}$  were from Aldrich.  $\text{LiAlH}_4$  was from Alfa (Ward Hill, MA); Scintiverse was from Fisher Scientific; Triton X-100 was from Pierce. Protoporphyrin IX and derivatives were from Porphyrin Products (Logan, UT). All other reagents were from Sigma and J. T. Baker. The rhodium chelate was synthesized by the procedure of Monson (24). [9,10- $^3\text{H}$ ]Octadecanoic acid was prepared by hydrogenation of *cis*-9-[9,10- $^3\text{H}$ ]octadecenoic acid (25); [9,10- $^3\text{H}$ ]octadecanol and [1- $^{14}\text{C}$ ]octadecanol were synthesized from the corresponding labeled fatty acid by  $\text{LiAlH}_4$  reduction. [1- $^{14}\text{C}$ ]- and [9,10- $^3\text{H}$ ]Octadecanol were oxidized to the aldehyde by using pyridinium chlorochromate (26).

***B. braunii*.** Strain A from the Austin Culture Collection (University of Texas) was grown in 20-liter clear carboys (Nalge). Sterile air enriched with 1%  $\text{CO}_2$  was sparged into the vessels at a rate of 5 liters/hr through ceramic air stones from a local aquarium store. The alga was grown in modified CHU 13 medium at  $2\times$  strength (21, 27) under cool-white fluorescent light at 25°C. Cultures were harvested between 9 and 12 days by centrifugation.

Abbreviation: DTE, dithioerythritol.

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**Solubilization of Aldehyde Decarbonylase.** *B. braunii* [25 g (wet weight)] was homogenized in 50 ml of 0.1 M potassium phosphate (pH 7.0) containing 0.3 M sucrose for 2 min by using a microhomogenizer. The homogenate was centrifuged for 1 min at  $2940 \times g$  in an Eppendorf microcentrifuge. The supernatant was centrifuged for 90 min at  $105,000 \times g$ , and the microsomal pellet was resuspended in 500  $\mu$ l of solubilization buffer containing 0.1 M potassium phosphate (pH 7.0), 2 mM ascorbate, 0.05 mM DTE, and 0.1% of one of the following detergents: Triton X-100, octyl  $\beta$ -glucoside, Nonidet P-40, or deoxycholate. After gentle shaking for 30 min at 25°C, the suspension was recentrifuged at  $105,000 \times g$  for 90 min. The clear supernatant was carefully removed, and the pellet was resuspended in 250  $\mu$ l of solubilization buffer containing the respective detergent. Both the supernatant and the resuspended pellet were assayed for decarbonylase activity. Protein was determined by the method of Bradford (28). All procedures were done at 4°C.

**Enzyme Purification.** *B. braunii* (10–12 days in culture) was homogenized as indicated above and the enzyme was solubilized with 0.1% octyl  $\beta$ -glucoside. Aliquots (200  $\mu$ l) of the solubilized decarbonylase were injected onto an FPLC Superose 6 HR 10/30 column (Pharmacia), previously equilibrated with 0.05 M potassium phosphate (pH 7.0) containing 2 mM ascorbate, 0.05 mM DTE, and 0.1% octyl  $\beta$ -glucoside. The proteins were eluted with the same buffer at a flow rate of 0.4 ml/min, and 0.5-ml fractions were collected. Fractions containing decarbonylase activity were pooled and injected onto an FPLC Mono Q HR 5/5 column (Pharmacia), which had been previously equilibrated with 0.05 M potassium phosphate (pH 7.0) containing 2 mM ascorbate, 0.05 mM DTE, and 0.1% octyl  $\beta$ -glucoside. The column was washed with one bed volume of the 0.05 M potassium phosphate (pH 7.0) and proteins were eluted with a 10-ml linear gradient of 0.0–1.0 M KCl. The column effluent was monitored at 280 nm, and 0.5-ml fractions were collected. Column fractions containing decarbonylase activity were pooled and concentrated by Centricon (Amicon) ultrafiltration. This preparation was used for all further studies on the enzyme.

**Enzyme Assays.** Alkane synthesis was assayed in a manner similar to that previously described (18). Assays were done anaerobically in 16  $\times$  100-mm test tubes containing three polypropylene cups with filter paper strips that were sealed with serum stoppers. One cup contained  $\text{RhCl}[(\text{C}_6\text{H}_5)_3\text{P}]_3$  to trap the enzymatically released CO. Another cup contained methylbenzethonium hydroxide to absorb any released  $\text{CO}_2$ ; this trap does not absorb CO. After flushing the reaction tubes for 2 min with  $\text{N}_2$ , 200  $\mu$ l of freshly prepared 12.5% (wt/vol) pyrogallol in 20% (wt/vol) KOH was injected into the third cup to remove any remaining  $\text{O}_2$ . The reaction mixtures were incubated for 5 min at 25°C before injection of the enzyme. Each reaction mixture contained 65  $\mu$ M [9,10- $^3\text{H}$ , 1- $^{14}\text{C}$ ]octadecanal, the octyl glucoside-containing solubilization buffer, and enzyme in a total volume of 2.0 ml. After incubation at 25°C for 45 min, 200  $\mu$ l of 2 M HCl was added. After photolysis the CO and  $\text{CO}_2$  traps were removed and assayed for  $^{14}\text{C}$  (17). The lipids extracted from the mixture were separated by TLC, and the isolated alkane fraction was assayed for radioactivity as described (17, 18). When needed, aliquots of the aqueous phase were assayed for radioactivity. The chain length of the alkane product was determined by radio-gas chromatography (23).

**Electrophoresis.** SDS/PAGE was carried out with a Hoefer model SE-400 apparatus. Analytical slab gels (10%) were formed with a 3% stacking gel, and a discontinuous buffer system was used (29). Proteins were silver-stained (30).

**UV/Visible Spectrophotometry.** An absorption spectrum of the decarbonylase preparation was determined on a Hewlett Packard model 8451A diode array spectrophotometer against the buffer blank.

**Electron Microprobe Analysis.** Enzyme samples (25  $\mu$ l containing  $\approx 4$   $\mu$ g of protein) were dried under vacuum into 2.0-cm-long grooves etched into glass plates. The analysis was carried out on a PDP LB3 French version of an American Digital instrument containing four spectrometers. The electron beam was set at 40  $\mu$ m and the detection limits for the metals were between 50 and 70 ppm. Background values obtained with the buffer were subtracted from the values obtained with the samples containing the decarbonylase.

**In Vivo  $^{57}\text{Co}$  Labeling of Decarbonylase.** *B. braunii* was grown in a 1.8-liter Fernbach flask behind lead shielding in modified CHU 13 medium containing 0.5 mCi of  $^{57}\text{CoCl}_2$  (1 Ci = 37 GBq) under cool-white light. Cells (3.2 g) were harvested after 10 days by centrifugation and all remaining enzyme preparations and chromatography were done as with the unlabeled decarbonylase. Fractions obtained from FPLC Superose 6 HR 10/30 and Mono Q 5/5 columns were assayed for  $^{57}\text{Co}$  on a Packard 5400 series Crystal II multidetector  $\gamma$  counter. Labeling was performed on two batches of *B. braunii*.

**Nonenzymatic Decarbonylation with a Co-Porphyrin.** Decarbonylation of 100  $\mu$ M [9,10- $^3\text{H}$ ; 1- $^{14}\text{C}$ ]octadecanal by Co-protoporphyrin IX chloride was performed by substituting 0.1 mM of the porphyrin for the decarbonylase in the enzyme assay as described above with the exception of temperature that was held at 30, 40, 60, or 80°C. Reactions were allowed to proceed for 45 min and the products, [ $^3\text{H}$ ]heptadecane and  $^{14}\text{CO}$ , were measured as described for the enzymatic assays. All other metal porphyrins examined for decarbonylation activity were at 0.1 mM and the reaction temperature was 60°C.

## RESULTS

**Solubilization of Aldehyde Decarbonylase.** Decarbonylase activity was found in particulate preparations from both plants and animals (17–19, 23). To elucidate the nature of the enzyme that catalyzes this reaction and to elucidate the mechanism of this reaction, the enzyme has to be solubilized and purified. A *B. braunii* microsomal preparation was found to be suitable for this purpose. Decarbonylase activity in the microsomes was highest after 10 days of algal growth. Therefore, in all decarbonylase purification experiments, *B. braunii* was harvested at  $10 \pm 2$  days. To test whether the aldehyde decarbonylase could be solubilized in an enzymatically active form, microsomal preparations were incubated with 0.1% Triton X-100, 0.1% octyl  $\beta$ -glucoside, 0.1% Nonidet P-40, or 0.1% deoxycholate. The decarbonylase activity found in the supernatant after centrifugation at  $105,000 \times g$  was considered to be due to the solubilized enzyme. Octyl  $\beta$ -glucoside gave considerably higher solubilized-enzyme activity than the other detergents; deoxycholate also solubilized a considerable portion of the decarbonylase. Octyl  $\beta$ -glucoside-solubilized enzyme had a specific activity 40-fold higher than the crude cell-free preparation. This detergent achieved a 65% solubilization of the enzyme. Therefore, all future solubilizations of aldehyde decarbonylase were performed using 0.1% octyl  $\beta$ -glucoside.

**Purification of Aldehyde Decarbonylase.** Upon FPLC Superose 6 gel filtration of the solubilized decarbonylase preparation from the microsomes, the decarbonylase activity was eluted in fractions at or near the void volume (Fig. 1). When the partially purified decarbonylase from the gel-filtration step was subjected to anion-exchange FPLC on a Mono Q HR 5/5 column, no aldehyde decarbonylase activity could be detected in the proteins that were not retained in the column. A linear increase of the ionic strength with KCl resulted in the elution of several proteins from the column. Decarbonylase activity was located in fractions corresponding to the second major protein peak. The solubilization and purification procedures used resulted in a  $>200$ -fold increase in specific activity of the decarbonylase with a 7% recovery of total

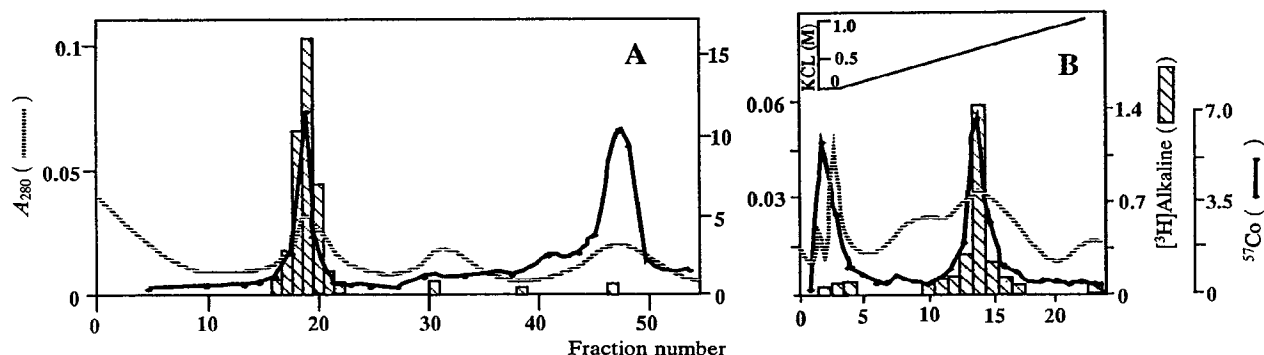


FIG. 1. Purification of aldehyde decarbonylase solubilized from microsomes of *B. braunii* and coelution of  $^{57}\text{Co}$  with the decarbonylase. (A) Gel filtration. Portions (200  $\mu\text{l}$ ) of the solubilized decarbonylase were injected onto an FPLC Superose 5 HR 10/30 column (Pharmacia) previously equilibrated with the solubilization buffer, the proteins were eluted at a flow rate of 0.4 ml/min, and 0.5-ml fractions were collected. (B) Ion-exchange chromatography. Fractions from the gel-filtration step that contain decarbonylase activity were pooled and injected onto an FPLC Mono Q HR 5/5 column (Pharmacia) that had been previously equilibrated with the solubilization buffer. After washing the column with one bed volume of the buffer, a linear gradient of 0.0–1.0 M KCl in a total volume of 10 ml of the same buffer was applied, and 0.5-ml fractions were collected. Radioactivity of  $[^3\text{H}]$ alkane is reported as dpm ( $\times 10^{-3}$ ) and radioactivity of  $^{57}\text{Co}$  is reported as cpm ( $\times 10^{-2}$ ).

activity (Table 1). SDS/PAGE of the purified decarbonylase preparations showed two silver-stained bands of similar intensities at 66 and 55 kDa (Fig. 2).

**Stoichiometry of Enzymatic Aldehyde Decarbonylation.** In view of the fact that, with crude microsomal preparations from *B. braunii*, very little CO could be recovered because of further metabolism of this product (23), CO evolution from the purified enzyme was examined. Both CO and  $\text{CO}_2$  were trapped during the decarbonylation of  $[9,10\text{-}^3\text{H}, 1\text{-}^{14}\text{C}]$ octadecanal with the purified enzyme. The CO released was near equimolar with the alkane produced; very little  $\text{CO}_2$  was observed; an average of 0.84 mol of CO was recovered for each mol of heptadecane formed.

**UV/Visible Spectra.** The absorbance spectrum of the aldehyde decarbonylase is shown in Fig. 3. Besides the normal protein absorbance at 260 and 280 nm, the enzyme showed absorbance peaks at  $\approx 410$  nm, 425 nm, 580 nm, and 620 nm. These peaks are highly indicative of a porphyrin moiety.

**Metals.** Since metal ion chelators are known to inhibit decarbonylase activity (17, 18, 23), we examined the purified enzyme for the presence of metal ion. Electron microprobe analyses of different aldehyde decarbonylase preparations consistently showed significant amounts of Co with much smaller and variable amounts of other metals (Table 2). On average 1536 ppm of Co was observed, which calculated to  $1.13 \times 10^{-11}$  mol of Co per  $\mu\text{g}$  of protein. To test whether Co really is the metal contained in the enzyme, *B. braunii* was grown in medium containing  $^{57}\text{CoCl}_2$  for 10 days. The microsomal preparation obtained from labeled cells was treated with octyl  $\beta$ -glucoside to solubilize the enzyme. The solubilized protein contained 50% of the  $^{57}\text{Co}$  contained in the microsomal preparation. Upon gel filtration on an FPLC Superose 6 HR 10/30 column, the elution profile of decarbonylase activity coincided with that of  $^{57}\text{Co}$  (Fig. 1). When

the  $^{57}\text{Co}$ -labeled decarbonylase preparation from the gel filtration was applied to an FPLC ion-exchange Mono Q column, a small amount of label emerged in the wash that contained no decarbonylase activity. Upon elution of decarbonylase activity by a KCl gradient,  $^{57}\text{Co}$  was coeluted with the enzyme activity (Fig. 1).

**Nonenzymatic Decarbonylation with a Co-Porphyrin.** In view of the report that a Ru-porphyrin can cause nonenzymatic decarbonylation (16), we tested whether a Co-porphyrin could also decarbonylate octadecanal. Coproporphyrin IX chloride decarbonylated  $[9,10\text{-}^3\text{H}]$ octadecanal optimally at  $60^\circ\text{C}$  but did not exhibit activity at ambient temperatures (Fig. 4). Neither protoporphyrin IX alone nor Co alone caused decarbonylation of octadecanal (Table 3). The metal chelators *o*-phenanthroline, 8-hydroxyquinoline, and EDTA inhibited decarbonylation by Co-porphyrin. Of the other metal porphyrins examined, Fe-protoporphyrin IX chloride showed 8% of the decarbonylation activity observed with Co-protoporphyrin IX chloride, whereas Mn-, Sn-, and Zn-protoporphyrin IX chlorides did not generate detectable amounts of alkane from octadecanal at  $60^\circ\text{C}$ .

## DISCUSSION

The mechanism of biosynthesis of hydrocarbons remained obscure for a long time in spite of the fact that this simple class of organic compounds are produced by a wide variety of living organisms (1). Although hydrocarbon synthesis was shown to involve the loss of the carboxyl carbon of chain-elongated fatty acid (9), direct loss of the carboxyl carbon of a fatty acid is not mechanistically feasible without an electron-withdrawing group adjacent to the  $\alpha$ -carbon. No intermediates with such a group has been detected. On the other hand experimental results suggested that an aldehyde could be the immediate precursor of a hydrocarbon (13, 14). The discovery of a

Table 1. Purification of decarbonylase from *B. braunii*

Step	Total protein, mg	Total nmol of alkane per min	Specific activity, nmol per min per mg	Apparent purification factor	Apparent yield, %
Crude	44.2	0.770	0.017	1.00	100
Microsomes	0.429	0.097	0.226	13.3	12.6
Octyl $\beta$ -glucoside	0.122	0.083	0.680	40.0	10.7
FPLC Superose 6	0.059	0.063	1.06	62.3	8.1
FPLC Mono Q	0.016	0.057	3.53	208	7.3

*B. braunii* (10 days old; 14.57 g) was used for this purification. The total decarbonylase activity in the microsomal fraction was much less than that in the crude extract. However, the  $105,000 \times g$  supernatant itself had very little activity. It is possible that the supernatant stimulated the microsomal enzyme.

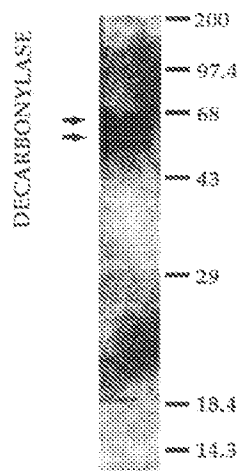


FIG. 2. SDS/PAGE of purified aldehyde decarbonylase. An analytical slab gel (10%) with a 3% stacking gel was used with the discontinuous buffer system of Laemmli (29) and the proteins were silver-stained by the procedure of Morrissey (30). Molecular masses in kDa are indicated.

chemical decarbonylation by Ru-porphyrin complex at elevated temperature provided a possible mechanism for conversion of an aldehyde to a hydrocarbon (15, 16). With the discovery of enzymes that catalyze conversion of an aldehyde to a hydrocarbon and CO, it became clear how the mechanistic difficulty of decarboxylating a fatty acid is circumvented by decarbonylating an aldehyde that was already known to be produced by microsomes by an acyl-CoA reductase (31).

The results presented in this paper show that the nonionic detergent octyl  $\beta$ -glucoside best solubilized the decarbonylase from the microsomal fraction. Octyl  $\beta$ -glucoside gave considerably higher activities than the other detergents used in this study, indicating possible stimulation of the enzyme as well as solubilization. The purification procedure resulted in a 200-fold increase in specific activity when compared to the crude cell-free preparation, and an apparent recovery of  $\approx 7\%$  of the original decarbonylase activity. SDS/PAGE revealed two protein bands of similar intensities at 66 and 55 kDa. Therefore, we tentatively conclude that the enzyme may have an  $\alpha_n\beta_n$  structure. Under the conditions so far used, the purified enzyme emerged at the void volume of Superose 6 and Sepharose 6B gel-filtration columns (data not shown) in the presence and absence of 0.1% octyl  $\beta$ -glucoside, probably indicating that the protein aggregated; therefore, the molecular weight of the native enzyme is not known.

Upon independent electron microprobe analyses of aldehyde decarbonylase preparations, the only metal that was found consistently in significant amounts was Co. The presence of Co in the enzyme is consistent with the previous findings that metal chelators such as EDTA,  $\alpha$ -phenanthro-

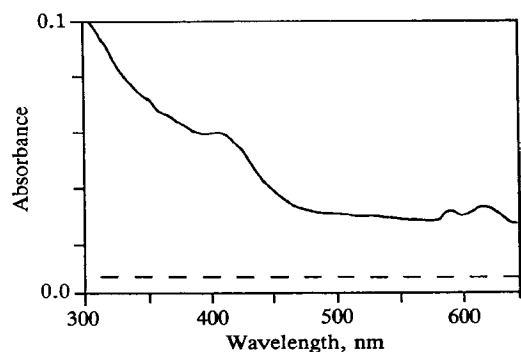


FIG. 3. Absorption spectrum of purified decarbonylase preparation (solid line). The spectrum was recorded in 0.05 M potassium phosphate, pH 7.0/2.0 mM ascorbate/0.05 mM DTE/0.1% octyl  $\beta$ -glucoside on an HP 8451AS diode array spectrophotometer. Buffer control showed no absorption (dashed line).

Table 2. Electron microprobe analysis of decarbonylase from *B. braunii*

Metal	Amount, ppm
Fe	0
Co	1536
Ni	156
Cu	192
Mg	0
Mn	259
Mo	0
Zn	351

Metal value reported is that in sample minus buffer control containing 0.05 M potassium phosphate (pH 7.0) and 0.1% octyl  $\beta$ -glucoside in triple-distilled deionized water.

line, and 8-hydroxyquinoline severely inhibited aldehyde decarbonylase from the plant and animal systems thus far examined (17, 18, 23). That the isotope  $^{57}\text{Co}$  copurifies with the decarbonylase activity upon gel-filtration and ion-exchange chromatography of enzyme preparations from cells grown in  $^{57}\text{CoCl}_2$  adds further evidence that a Co ion is a component of the decarbonylase. With the assumption that aldehyde decarbonylase is a heterodimer consisting of a 66-kDa monomer and a 55-kDa monomer, the metal content of the enzyme was calculated to be 1.37 mol of Co per mol (121 kDa) of protein.

In addition to the normal absorbance in the 260- and 280-nm region for a protein, the aldehyde decarbonylase exhibited absorbance peaks at 410 nm, 425 nm, 580 nm, and 620 nm, suggesting the presence of a porphyrin. Porphyrins are known to absorb in the 380- to 420-nm region (Soret bands), and in the 500- to 600-nm region (the Q-bands) (32). The absorbance spectrum of a pure Co-protoporphyrin IX chloride shows four absorbance peaks at 350 nm, 410 nm, 535 nm, and 570 nm. Considering the fact that porphyrin absorbance usually shifts to the higher wavelength upon binding to the apoprotein (33), the absorption spectrum of the decarbonylase is consistent with the presence of a Co-porphyrin within the enzyme. By assuming that 1 mol (121 kDa) of enzyme contains 1 mol of porphyrin, an extinction coefficient for aldehyde decarbonylase at 410 nm was determined to be  $3.33 \times 10^4$  liter $\cdot$ cm $^{-1}$  $\cdot$ mol $^{-1}$ . This value is in the same range as

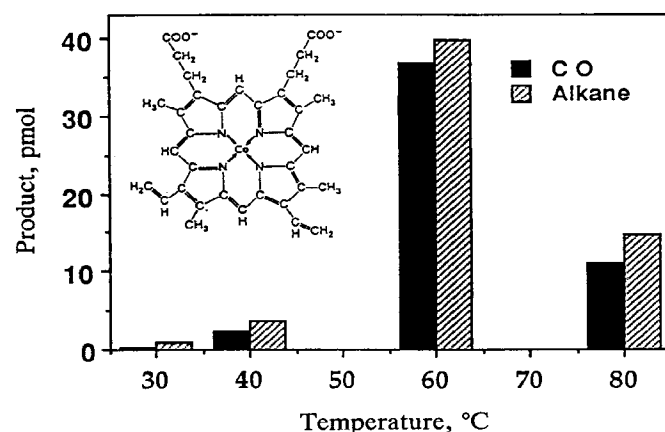


FIG. 4. Decarbonylation of octadecanal by Co-protoporphyrin IX. Decarbonylation was measured with 100  $\mu\text{M}$  [9,10- $^3\text{H}$ ; 1- $^{14}\text{C}$ ]octadecanal and 0.1 mM Co-protoporphyrin IX, as was done for the enzymatic reaction at indicated temperatures. Reactions were allowed to proceed for 45 min and the products, [ $^3\text{H}$ ]heptadecane and  $^{14}\text{CO}$ , were quantitated as described for the enzymatic assays. All other metal porphyrins examined for decarbonylation activity were at 0.1 mM and the reaction temperature was 60°C.

Table 3. Effects of metal chelators on nonenzymatic decarbonylation with Co-protoporphyrin IX chloride and a comparison of activity with other metal porphyrins at 60°C

Addition(s)	Alkane, pmol	CO, pmol
Co-protoporphyrin IX chloride	28	20
+ EDTA (10.0 mM)	0	0.5
+ <i>o</i> -Phenanthroline (1.0 mM)	2.8	6.0
+ 8-Hydroxyquinoline (1.0 mM)	0	0
CoCl <sub>2</sub> (alone)	0	0.9
Protoporphyrin IX (alone)	0	0
Fe-protoporphyrin IX chloride	2.1	2.1
Mn-protoporphyrin IX chloride	0	0.8
Sn-protoporphyrin IX dichloride	0	1.0
Zn-protoporphyrin IX	0	1.0

Alkane and CO were measured as pmol generated above the background control reaction with [9,10-<sup>3</sup>H, 1-<sup>14</sup>C]octadecanal alone.

many known porphyrin-containing proteins (33). From this result and the Co content of the enzyme indicated above, we tentatively concluded that the decarbonylase consists of one Co-porphyrin per  $\alpha\beta$  pair of subunits. Even though the spectrum resembles that of porphyrin more than that of corrin, definite identification of the tetrapyrrole in the enzyme as a porphyrin must await further physical chemical and chemical characterization that would require quantities of enzyme not currently available.

The evidence that suggests that the aldehyde decarbonylase contains a Co-porphyrin prompted us to explore whether Co-porphyrin has the inherent capability to cause nonenzymatic decarbonylation. In fact, Co-porphyrin IX chloride catalyzed the decarbonylation at 60°C, whereas the individual constituents of the Co-protoporphyrin IX chloride did not cause decarbonylation. Metal ion chelators, known to inhibit the enzymatic decarbonylation, also inhibited the nonenzymatic decarbonylation by Co-porphyrin. The other metal porphyrins explored in this study showed no decarbonylation with the exception of Fe-protoporphyrin IX chloride, which decarbonylated octadecanal at 8% of the rate observed with the Co-protoporphyrin IX chloride. That this Co-porphyrin can catalyze the same reaction as aldehyde decarbonylase at higher temperatures demonstrates the inherent capacity of the prosthetic group in the decarbonylase to participate in the reaction catalyzed by the enzyme. Obviously, the apoprotein enhances the basic capability of the prosthetic group to make decarbonylation possible at the biological temperature. Co has very similar coordination chemistry to Ru, and ruthenium diphosphine ligands are known to chemically decarbonylate aldehydes to hydrocarbons at elevated temperatures (15). It is probable that the decarbonylase enzyme with its Co-porphyrin catalyzes the decarbonylation by using a mechanism similar to that suggested for the decarbonylation by the rhodium diphosphine ligands at elevated temperatures (34).

The purified aldehyde decarbonylase catalyzed the decarbonylation of octadecanal to heptadecane and CO with near 1:1 stoichiometry. The crude particulate fraction from which the decarbonylase was obtained did not show such a stoichiometry (23), because this preparation also catalyzed the conversion of CO to CO<sub>2</sub> with subsequent metabolism of CO<sub>2</sub> into other cellular components. Thus, the alga is able to utilize the normally toxic CO produced by the decarbonylation. Such a detoxification was not observed with the particulate preparations from the pea leaves (17). Since the decarbonylase appeared to be present in association with the cuticle outside the cell, CO generated would probably escape into the atmosphere without any adverse effects on the plant. Similarly, in the uropygial gland of the grebe, CO probably would escape

through the secretory channels from this holocrine gland (18) and thus may not require a detoxification mechanism.

Co has been known for a long time to be an essential element for plant growth. However, to our knowledge, enzymic reactions involving Co have not been elucidated in plants until now. Considering the omnipresence of hydrocarbons in plants and their role in waterproofing to prevent desiccation, it is probable that one important function of Co in plants is its role in decarbonylation. Co-porphyrins might be of wider occurrence. There are several reports on Co-porphyrin-containing proteins in bacteria (35–37), but such proteins have not been previously detected in plants.

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Wednesday, June 06, 2007

## Building Better Biofuels

Startup LS9 is developing microbes that produce hydrocarbons.

By Neil Savage

The U.S. Department of Energy has set a goal of replacing 30 percent of gasoline used in the United States with fuels from renewable biological sources by 2030, and President Bush has made ethanol production a priority. So it is hardly surprising that some biotech startup companies are positioning themselves to take advantage of an anticipated booming market for biofuels.

While much of the focus is on ethanol, [LS9](#), of San Carlos, CA, is using the relatively new field of synthetic biology to engineer bacteria that can make hydrocarbons for gasoline, diesel, and jet fuel. Hydrocarbon fuels are better suited than ethanol to existing delivery infrastructure and engines, and their manufacture would require less energy. To make biological production of hydrocarbons a reality, the company is bringing together leaders in synthetic biology and industrial biotechnology.

LS9 is at a very early stage: the company was formed in 2005, but its existence was announced only this winter. It plans to engineer microbes to incorporate gene pathways that other microbes, plants, and even animals use to store energy. Other startups, such as [Amyris](#), of Emeryville, CA, and [SunEthanol](#), of Amherst, MA, are also trying to use synthetic biology to develop microorganisms that produce biofuels. Stephen del Cardayre, a biochemist and LS9's vice president for research and development, says LS9 microbes produce and excrete hydrocarbons that are useful as fuels.

Now the company is working to customize the rate of production and the products themselves. "We certainly have gone beyond what we think anybody else was even thinking of doing" in terms of producing hydrocarbons from microbes, says George Church, a geneticist at Harvard Medical School and one of LS9's two founders. The other is Chris Somerville, professor of plant biology at Stanford University.

The company has \$5 million in funding from [Khosla Ventures](#), of Menlo Park, CA, and [Flagship Ventures](#), of Cambridge, MA. Its acting CEO, Douglas Cameron, is former director of biotechnology research at Cargill and chief scientific officer at

Khosla Ventures. Flagship CEO Noubar Afeyan cautions that no one can tell the extent to which any biofuel will displace fossil fuels. "That is a subject of great debate and great prognostication," he says. "The opportunity is so large that I don't have to believe in much more than a few percentage points of market penetration for it to be worth our investment."

The company is looking for areas where synthetic biology's potential to produce specific types of molecules will pay off. This could mean making high-performance jet fuel, Afeyan says, or it could mean creating gasoline that has no pollution-causing sulfur content. Beyond custom-developing hydrocarbons, LS9 foresees licensing its technology. In particular, the company might someday forge agreements with ethanol producers, whose manufacturing plants could be put to more profitable and efficient use making hydrocarbon fuels.

LS9 is counting on the fact that ethanol is not really the best biofuel. Del Cardayre notes that ethanol can't be delivered through existing pipelines. It also contains 30 percent less energy than gasoline, and it must be mixed with gasoline before being burned in conventional engines. LS9's fuels would have none of these disadvantages. What's more, LS9's fuels might be produced more efficiently than ethanol. For example, at the end of ethanol fermentation, the mixture has to be distilled to separate ethanol from water. LS9's products would just float to the top of a fermentation tank to be skimmed off. Overall, the LS9 process consumes about 65 percent less energy than today's ethanol production, the company says.

LS9 now needs to prove that its technology is economical and can produce fuels on a large scale, says Jim McMillan, principal biochemical engineer in the National Renewable Energy Laboratory's Bioenergy Center, based in Golden, CO. "I don't doubt that [making hydrocarbon fuel from microbes] can be done; the question is how quickly and at what cost," he says. LS9 says it hopes to bring its hydrocarbon biofuels to market in four or five years.

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## **Upcoming Events**

EmTech08

MIT Campus, Cambridge, MA

Tuesday, September 23, 2008 - Thursday, September 25, 2008

<http://www.technologyreview.com/emtech/08/>

**Nano-Net 2008**

Boston, MA

Monday, September 15, 2008 - Thursday, September 18, 2008

<http://www.nanonets.org>

**NanoMedicine Summit**

Cleveland, OH

Thursday, September 25, 2008 - Friday, September 26, 2008

<http://www.nanomedicinesummit.org>

**WHIT 4.0**

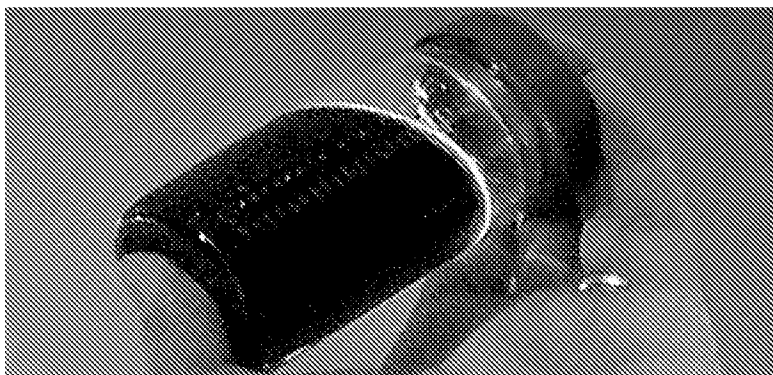
Washington, DC

Monday, December 08, 2008 - Wednesday, December 10, 2008

<http://www.whitcongress.com>

## Scientists find bugs that eat waste and excrete petrol

Silicon Valley is experimenting with bacteria that have been genetically altered to provide 'renewable petroleum'



Some diesel fuel produced by genetically modified bugs

Chris Ayres

"Ten years ago I could never have imagined I'd be doing this," says Greg Pal, 33, a former software executive, as he squints into the late afternoon Californian sun. "I mean, this is essentially agriculture, right? But the people I talk to – especially the ones coming out of business school – this is the one hot area everyone wants to get into."

He means bugs. To be more precise: the genetic alteration of bugs – very, very small ones – so that when they feed on agricultural waste such as woodchips or wheat straw, they do something extraordinary. They excrete crude oil.

Unbelievably, this is not science fiction. Mr Pal holds up a small beaker of bug excretion that could, theoretically, be poured into the tank of the giant Lexus SUV next to us. Not that Mr Pal is willing to risk it just yet. He gives it a month before the first vehicle is filled up on what he calls "renewable petroleum". After that, he grins, "it's a brave new world".

Mr Pal is a senior director of LS9, one of several companies in or near Silicon Valley that have spurned traditional high-tech activities such as software and networking and embarked instead on an extraordinary race to make \$140-a-barrel oil (£70) from Saudi Arabia obsolete. "All of us here – everyone in this company and in this industry, are aware of the urgency," Mr Pal says.

What is most remarkable about what they are doing is that instead of trying to reengineer the global economy – as is required, for example, for the use of hydrogen fuel – they are trying to make a product that is interchangeable with oil. The company claims that this "Oil 2.0" will not only be renewable but also carbon negative – meaning that the carbon it emits will be less than that sucked from the atmosphere by the raw materials from which it is made.

LS9 has already convinced one oil industry veteran of its plan: Bob Walsh, 50, who now serves as the firm's president after a 26-year career at Shell, most recently running European supply operations in London. "How many times in your life do you get the opportunity to grow a multi-billion-dollar company?" he asks. It is a bold statement from a man who works in a glorified cubicle in a San Francisco industrial estate for a company that describes itself as being "prerevenue".

Inside LS9's cluttered laboratory – funded by \$20 million of start-up capital from investors including Vinod Khosla, the Indian-American entrepreneur who co-founded Sun Micro-systems – Mr Pal explains that LS9's bugs are single-cell organisms, each a fraction of a billionth the size of an ant. They start out as industrial yeast or nonpathogenic strains of *E.*

*coli*, but LS9 modifies them by custom-de-signing their DNA. "Five to seven years ago, that process would have taken months and cost hundreds of thousands of dollars," he says. "Now it can take weeks and cost maybe \$20,000."

Because crude oil (which can be refined into other products, such as petroleum or jet fuel) is only a few molecular stages removed from the fatty acids normally excreted by yeast or *E. coli* during fermentation, it does not take much fiddling to get the desired result.

For fermentation to take place you need raw material, or feedstock, as it is known in the biofuels industry. Anything will do as long as it can be broken down into sugars, with the byproduct ideally burnt to produce electricity to run the plant.

The company is not interested in using corn as feedstock, given the much-publicised problems created by using food crops for fuel, such as the tortilla inflation that recently caused food riots in Mexico City. Instead, different types of agricultural waste will be used according to whatever makes sense for the local climate and economy: wheat straw in California, for example, or woodchips in the South.

Using genetically modified bugs for fermentation is essentially the same as using natural bacteria to produce ethanol, although the energy-intensive final process of distillation is virtually eliminated because the bugs excrete a substance that is almost pump-ready.

The closest that LS9 has come to mass production is a 1,000-litre fermenting machine, which looks like a large stainless-steel jar, next to a wardrobe-sized computer connected by a tangle of cables and tubes. It has not yet been plugged in. The machine produces the equivalent of one barrel a week and takes up 40 sq ft of floor space.

However, to substitute America's weekly oil consumption of 143 million barrels, you would need a facility that covered about 205 square miles, an area roughly the size of Chicago.

That is the main problem: although LS9 can produce its bug fuel in laboratory beakers, it has no idea whether it will be able to produce the same results on a nationwide or even global scale.

"Our plan is to have a demonstration-scale plant operational by 2010 and, in parallel, we'll be working on the design and construction of a commercial-scale facility to open in 2011," says Mr Pal, adding that if LS9 used Brazilian sugar cane as its feedstock, its fuel would probably cost about \$50 a barrel.

Are Americans ready to be putting genetically modified bug excretion in their cars? "It's not the same as with food," Mr Pal says. "We're putting these bacteria in a very isolated container: their entire universe is in that tank. When we're done with them, they're destroyed."

Besides, he says, there is greater good being served. "I have two children, and climate change is something that they are going to face. The energy crisis is something that they are going to face. We have a collective responsibility to do this."

### **Power points**

- Google has set up an initiative to develop electricity from cheap renewable energy sources
- Craig Venter, who mapped the human genome, has created a company to create hydrogen and ethanol from genetically engineered bugs
- The US Energy and Agriculture Departments said in 2005 that there was land available to produce enough biomass (nonedible plant parts) to replace 30 per cent of current liquid transport fuels

# PLENTY

IT'S EASY BEING GREEN

## Next-generation biofuels: nearly identical to gasoline and diesel

Existing infrastructure—including refineries and pipelines—will be able to handle biocrude and biodiesel, allowing for easy adoption, say biotech companies

By Melinda Wenner



The biofuel industry has had a bumpy few years: Ethanol is still a Department of Energy golden child, but recent studies raise serious questions about its viability. Production could eat up half of America's corn crop this year, potentially causing food shortages, and some say that ethanol manufacturing uses more energy than it produces. What's more, the fuel's corrosiveness makes it unsuitable for distribution via existing petroleum pipelines. But two new biotech companies believe biofuels can leapfrog past these problems, and they're each engineering fuels that are virtually identical to the gasoline and diesel we use today.

"We said, 'What does nature make that looks like a fuel?'" explains Kinkead Reiling, cofounder of Amyris Biotechnologies in Emeryville, California. Every living organism efficiently converts sugar from food into fat to store energy, and fats are chemically similar to the hydrocarbons that make up fuels. So by tinkering with a few genes, scientists at Amyris and San Carlos, California, biotech firm LS9 have designed bacteria that eat almost any type of plant—mostly sugarcane, corn, and other forms of cellulose—digest it, and convert it into "fuel."

The companies' plans are feasible because of how quickly and cheaply scientists can now sequence and synthesize genes. It took thirteen years and \$3 billion to order the first human genome, but last year, Nobel Laureate James Watson had his own sequenced in two months for less than \$1 million. Amyris and LS9 isolated and arranged the genes

necessary to make petroleum-like fuels in 2005 and 2007, respectively, and introduced those genes into bacteria, creating billions of live biofuel factories for a fraction of what it would have cost five years earlier. “Because we have this genetic control over the organisms, we can really tailor the set of molecules that come out the other side,” says Greg Pal, LS9’s senior director of corporate development.

Amyris and LS9 are producing fuels that are slightly different from one another. Amyris is making gasoline and diesel similar enough to conventional forms that they can be pumped through existing pipelines and directly into cars. In doing so, the company, which raised \$20 million in 2006 and another \$70 million last year, could bypass one of biofuel’s biggest problems in the short term: distribution.

Whereas Amyris is creating a product similar to conventional refined fuels, LS9 is touting the real deal. “If you took all the constraints off, what would you make?” Pal asks. The answer: crude oil. LS9, which secured \$15 million in phase two funding last year and was recognized by the World Economic Forum as one of the 39 Technology Pioneers of 2008, is engineering bacteria to make crude that can be shipped directly to refineries.

Conventional crude oil can contain thousands of types of molecules—that’s why it’s called “crude”—but LS9’s will have only about ten, which Pal says is ideal: The company’s oil will have the molecular diversity necessary to make a number of fuels and petroleum products but will be free of unwanted chemicals that can muck up engines. It’s “a pretty optimal solution,” he says.

The magic year for both companies is 2011, when LS9 expects its crude oil to be in full-scale production, and Amyris’ diesel, which solidifies at a lower temperature than vegetable-oil biodiesel, should come to market, too. Amyris’ jet fuel and gasoline and an LS9 biodiesel will follow several years later.

What’s so green about fuels practically identical to conventional ones? The answer lies in how they’re made. These newer biodiesels start out as plants that suck carbon dioxide out of the air, so burning them releases little, if any, net CO<sub>2</sub>—it’s a closed loop, Pal argues. LS9 estimates that its bacteria produce 90 BTUs of fuel for every 100 BTUs of sugar they eat. And although genetically engineered, the bacteria are not producing something completely synthetic. “The molecules we’re creating are made in nature by plants and organisms,” Reiling says. “We’re just changing the setting in which they’re made.”

Posted on May 21, 2008 at 7:24 AM